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DISSOLUTION OF HYDROXYLAPATITE BY SOIL BACTERIA: METHODS OF STUDY AND FATE OF THE DISSOLVED PHOSPHORUS

by

SABTI MOHAMMAD HMEIDAN

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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DEDICATION

TO MY COUNTRY,"PALESTINE".

ABSTRACT

Phosphorus release into solution from sparingly soluble phosphate minerals by phosphate dissolving bacteria (PDB) has been studied in the past, but no reports are available of attempts to evaluate the importance of PDB in pumping phosphorus from sparingly soluble minerals into microbial phosphorus.

In this study, solubilization of $Ca_{10}(OH)_2(PO_4)_6$ or hydroxlapatite (HA) by PDB in both buffered and unbuffered systems was investigated. Separation of bacterial cells from undissolved HA particles was accomplished using 0.5N HCl solution. Partitioning of P between inorganic P in solution (Psi), organic P in solution (Pso) and biomass P (Pc) was examined in a liquid medium.

In unbuffered systems, a highly significant (P=0.01) inverse relationship ($r^2 = 0.94$) was found between $\ln[H_2PO_4^-]$ in solution and the final pH, indicating that pH was the main factor controlling the variability of P in solution. The final pH of the medium was influenced by the medium composition as well as the organisms. The correlation ($r^2=0.36$) between final pH and the total amount of P dissolved (Psi+Pso+Pc) was very much lower. This reflects, in part, the independent effect of P incorporation into biomass. The capacity of PDB to dissolve HA was independent of the soluble P content of the media.

In a system buffered with $CaCO_3$, the amount of orthophosphate in solution was negligible while a

significant amount of P was incorporated into microbial biomass. These data suggest an additional dimension to the role of PDB in P release from sparingly soluble phosphate minerals in buffered systems such as soils by converting it into organic P which may be released in a more available form following cell death. The microorganisms act as nutrient sources and sinks as well. The results also suggest that PDB affect the flow of both inorganic and organic P-forms, particularly the labile segments.

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1. INTRODUCTION

Phosphorus cycling in soil-plant systems is a complex process involving microorganisms, plant roots, soil organic P, soil minerals, soil inorganic P and the soil solution. Microorganisms influence the transformation of P through the mineralization of organic P, possibly altering the environment of sparingly soluble phosphate minerals, and by the immobilization of inorganic phosphorus. The process of microbial solubilization of sparingly soluble phosphate minerals has been stressed as a result of expected shortages of commercial fertilizers and increased prices of energy.

Bacteria and fungi capable of accelerating dissolution of sparingly soluble phosphate minerals have been isolated from soils, and from rhizospheres and rhizoplanes of many plants. Their ability to bring phosphorus into solution has been examined over the past 30 years. Eighty percent of the total microbial population of the soil appears capable of affecting the solubility of soil phosphate minerals. This percentage is usually higher in the rhizosphere and rhizoplane of plants than in the control soil.

Although increases in P concentration in solution by phosphate dissolving microorganisms (PDM) have been reported, no measurements have been made of the possible role of PDM in increasing the microbial and labile organic P pools through solubilization of sparingly soluble phosphate minerals. These PDM are able to accelerate the slow solubilization of sparingly soluble P-minerals. To date,

most studies with PDM have used *in vitro* techniques with pure cultures and various phosphate minerals as substrates in unbuffered systems. Generally, it has been assumed that the end-products of microbial metabolism, such as organic acids, were the main agents of solubilization of these minerals through either formation of stable complexes with divalent metals, or lowering of the pH of the medium.

Although PDM may markedly increase solution P concentration in unbuffered systems, the argument that soil is too highly buffered with respect to pH to permit such increase in P concentration in the soil solution, is frequently considered sufficient to negate acceptance of their activities as significant contributors to P cycling in soil-plant systems. Further, the data on the chelation concept is inadequate to provide a convincing argument for an important role of such a mechanism. If, however, these organisms pump P into a labile organic form, they may have an unsuspected role in P cycling. To date, no published studies have addressed this aspect of their activity.

The objectives of this study were to :

1. Develop a rapid and reliable method for isolation of PDB from soil.
2. Develop a method to trace the fate of P dissolved from sparingly soluble phosphate minerals.
3. Study the partitioning between organic and inorganic forms of P removed from phosphate minerals during the growth of PDB in buffered and unbuffered systems.

4. Examine the effects of soluble phosphate and of nitrogenous constituents of media on the solubilization of sparingly soluble phosphate minerals.

2. Literature Review

2.1 Phosphate Compounds in Soil

More than 90 % of the phosphorus in any ecosystem is found in non-living forms. The insoluble phosphate usually comprises about 95-99% of the total soil phosphate, and must be converted to directly available phosphorus before plants and microorganisms can benefit from it.

The insoluble phosphate in soil can be divided into two main categories, both of which are important sources of phosphorus to plants. These two categories are:

1.INSOLUBLE INORGANIC PHOSPHATES.

The inorganic phosphate in all soils is found attached to calcium (mainly in alkaline soil) or aluminum and iron (mainly in acidic soil) (Alexander 1973). The most common forms of calcium phosphate in soils listed in order of increasing solubility are:

Fluorapatite $3Ca_3(PO_4)_2.CaF$

Carbonateapatite $3Ca_3(PO_4)_2.CaCO_3$

Hydroxylapatite $3Ca_3(PO_4)_2.Ca(OH)_2$

Oxyapatite $3Ca_3(PO_4)_2.CaO$

Tricalcium phosphate $Ca_3(PO_4)_2$

Dicalcium phosphate $CaHPO_4$

Monocalcium phosphate $Ca(H_2PO_4)_2$ (Brady 1974).

Dicalcium and monocalcium phosphate are readily available to plants , but their quantity at any time is very

small because they revert to more insoluble forms. Fluorapatite is the most insoluble of the above group and is found even in the most weathered soils, especially in lower horizons (Brady,1974). Fluorapatite is weathered in soil and yields secondary materials,such as hydroxylapatite,which is more soluble than fluorapatite, but less soluble than oxyapatite and tricalcium phosphate (Hayman, 1975).

The iron and aluminum phosphates which are predominant in acid soil include hydrated phosphate, such as Strengite ($FePO_4.2H_2O$) , Vivianite ($Fe_3(PO_4)_2.8H_2O$), Variscite ($AlPO_4.2H_2O$), K-Tarankite ($H_6K_3Al_5(PO_4)_8.18H_2O$), and NH_4-Tarankite ($H_6(NH_4)_3Al_5(PO_4)_8.18H_2O$) (Lindsay, 1977). These latter two compounds are stable in acid soils and are extremely insoluble. In acid soils, phosphate is precipitated on the surface of iron and aluminum oxides, or by aluminum and iron ions either free in solution, or attached to silicate crystals such as kaolinite and montmorillonite. These processes are sometimes called "Phosphate Fixation" (Hayman,1975). Adding lime to acid soils,however, causes the (OH^-) ions to exchange with the H_2PO_4 ions in hydroxylapatite, and increases the availability of phosphorus. In general, calcium phosphate is more stable than aluminum and iron phosphate above pH 6-7; whereas, below pH 6-7 aluminum and iron phosphate is more stable than calcium phosphate (Hsu and Jackson,1960).

2. ORGANIC PHOSPHATES.

Organic phosphate in soil represents 15-85% of the total phosphorus . It is usually related to the nitrogen and carbon content of the soil. McGill and Cole (1981),however, question if there is a constant stoichiometric relation between nitrogen and phosphorus in soil organic matter. About 30-50% of organic phosphate consists of phytin material,largely inositol hexaphosphate (Hayman,1975). Half or less of the total occurs in soil as complexes with humic material. Microorganisms through mineralization processes, convert organic phosphate to readily available ortho-phosphate and assimilate part into microbial biomass.

Studies of the complete P cycle depend on rigorous evaluation of all the informational requirements for each of the constituent processes of the system. A critical point,however, in the study of P cycling is that the whole system must be studied and consideration of soil organic P cycling is meaningless by itself unless the soil inorganic P changes are simultaneously measured (Stewart et al,1980). Stewart and McKercher,(1981) as cited by Coleman *et al* (in press) showed the very important role which primary decomposers and grazers upon them play in phosphorus cycle (fig 1).

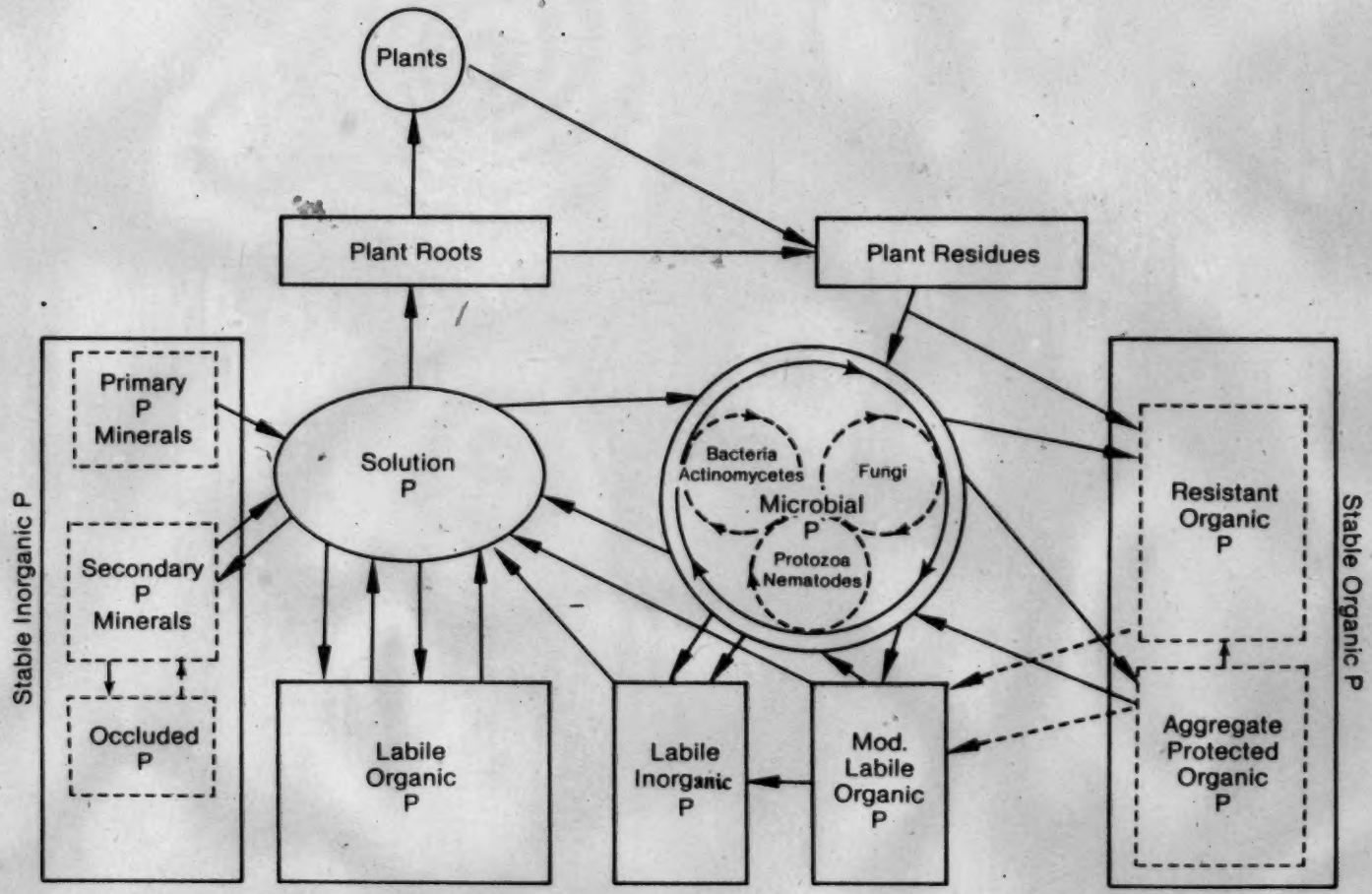

Figure 1: Flow chart of inorganic and organic forms of phosphorus (from Stewart and Hedley, in press).

2.2 Solubilization of Insoluble phosphate minerals.

2.2.1 Effect of Organic Acids.

Organic acids are normal constituents of most agricultural soils. They differ quantitatively and qualitatively from soil to soil , and in the same soil at different locations and times. In general, organic acids are present near decomposable plant materials and in the rhizosphere of plants. They are present in root exudates, as well as in leachates from surface litter horizons in forest soils. Also, they are produced as a result of the biological activities of microorganisms.

Some of the organic acids in soils have phytotoxic properties (Lynch,1978), while another group has a stimulative effect on the growth of higher plants (Vaughan and Malcolm,(1979);and Linehan, 1977). The pedogenic effect of microorganisms may be due,at least in part, to metabolically produced organic acids (Stevenson,1967).

According to Bracker (1955), bacteria and fungi differ to some extent in the nature of the organic acids produced. Volatile acids, such as formic,acetic,propionic and butyric are produced mainly by bacteria.Nonvolatile acids, such as citric and oxalic acids, are produced by many soil bacteria, as well as by fungi.(Stevenson,1967)

Sugar acids,such as gluconic, glucuronic and galacturonic, are produced in soil by several species of bacteria and fungi. Bacteria associated with rock surfaces

and soil microhabitats rich in organic matter produce 2-Ketogluconic acid in large quantities.

Aromatic acids such as ferullic,vanillic,syringic and p- hydroxybenzoic, have been detected in culture solutions from lignin decomposed by white rot fungi (Henderson 1955 and 1957 ;Hurst and Burges,1967; Martin and Haider,1971; Martin *et al*,1979). Flaig *et al* (1975) also reported that several microbes can synthesise phenolic and aromatic compounds.

Lichens are known to produce several acids,such as orsellic and B-orsellic as well as fatty straight chain acids (Hale,1961). These acids are able to form metal-complexes and to cause weathering of silicate minerals and to release Fe,Al,Ca and Mg into solution. (Iskander and Syers,1972).

Solubilization of native and added phosphorus in soil may be caused by a variety of mechanisms:-

1. Lowering the pH with organic acids or inorganic acids produced in soil as a result of microbal activity (Agnihotri, 1970).
2. Chelation of calcium,iron and aluminum by organic acids (Duff *et al*,1963 ;Duff and Webley,1959; Sperber,1958).
3. Formation of phosphohumic complexes (Bradly and Sieling,1953; and Sinha,1972).
4. Competition between humate and phosphate ions for absorbing surfaces (Swaby and Sperber,1958;Cosgrove,1977).

5. Production of hydrogen sulfide which reacts with the ferric phosphate precipitating iron as ferrous sulfide and in the process liberating phosphate,as in the following reaction:

$$2FePO_4 + 3H_2S = 2FeS + 2H_3PO_4 + S^\circ$$

(Sperber,1958a; Swaby and Sperber, 1958; Ehrlich,1981).

Struther and Sieling (1950) examined the influence of organic anions on the release of phosphate from iron and aluminum phosphate at different pH levels. Citrate, oxalate, tartrate and malate were most effective in preventing precipitation of phosphate by iron or aluminum. These anions were also found to be produced in greatest quantities in soil during the decomposition of organic matter (Shorey, 1913). Adding readily decomposable organic matter was more effective than the addition of a phosphate fertilizer in improving plant growth and phosphorus uptake (Neilson, 1953; Dalton, 1952). This was explained by the dissolution effect of organic materials produced by microbial action on organic matter. These organic substances were believed to form stable complex molecules with Al, Fe and Ca (Swensen *et al*, 1949; Schnitzer and Skinner, 1967; Sinha, 1972). Swensen (1949) ,Johnston (1952,1954) and Stevenson (1977) reported that the most effective acids in solubilizing phosphate minerals are those capable of forming stable complexes with Al, Fe and Ca. Zunino and Martin (1977) found that increasing the stability of complexes formed with soil organic matter would increase the role of organic matter in

soil formation and plant nutrition. They hypothesized a chain of chemical reactions whereby the metal would be translocated through the following phases :

Primary minerals, Parent material ----- soil colloidal particles i.e. soil organic matter and clays ----- soil solution ----- vegetal sap and tissues; microbial cytoplasm ----- blood plasma and tissues of higher animals.

They also found that humic acid-like substances and polysaccharides, synthesized by several orgamisms, have a high ability to absorb metallic cations.

Johnston (1959) found that organic acids,such as citric,oxalic and malic were much more effective than hydrochloric acid in dissolving iron and alluminium phosphate. This was supported by the findings of Mishustin (1972) as cited by Hayman (1975), who reported that chelation may be more important than a simple pH effect. Oxalic acid,which is a good chelator, dissolved more phosphate at pH 4.4 than did gluconic acid, a poor chelator, at pH 4.3 (Mishustin (1972), as cited by Hayman,1975).

The effect of fulvic acid and other humic substances on phosphate minerals, was studied by Sinha (1972). He concluded that these materials brought phosphate into solution. He explained the effect of these substances by the formation of stable fulvic acid/iron complexes. He also postulated the formation of fulvic acid/metal phosphate complexes and of a phosphohumic complex. The phosphorus in

these complexes was considered available to plants.

Another mechanism was suggested to explain the effect of organic substances on phosphate minerals. Barbier *et al* (1951),as cited by Stanford and Pierre (1953), proposed a competition between humate and phosphate ions for seats on adsorbing surfaces. Organic acids can also be adsorbed by clay minerals,such as kaolinite and on oxide surfaces, thereby decreasing the number of sites able to adsorb phosphate.

The efficiency of rock phosphate as a source of phosphate may be increased by the addition of sucrose,glucose, starch and pectin,apparently due to organic compounds produced from oxidation of carbohydrate material in soils. The addition of oxalic acid to soil, at rates of 270 and 540 kg ha^{-1}, increased the amount of native and added phosphate taken up by the plants (Steckel and Sieling,1965),as cited by Stevenson (1967).

The relationship between pH and dissolution of phosphate minerals has been widely studied and the following cases observed:-

1. A decrease in pH was accompanied by dissolution of phosphate minerals, with a negative correlation between degree of solubilization and pH (Swaby and Sperber,1958; Agnihorti,1970).
2. A decrease in pH was accompanied by dissolution of phosphate minerals,but there was no correlation between the degree of solubilization and pH (Sperber,1958a and

1958b).

3. In some cases the fall in pH was not accompanied by solubilization (Chhonkar and Subba-Rao,1967; and Paul and Sundara-Rao,1971).
4. Solubilization of phosphate minerals was not accompanied by a decrease in pH (Sperber,1958a).
5. There was a depression of solubilization under acid conditions; whereas,there was an increase in solubilization under alkaline conditions (Goswami and Abhiswas,1962).

In all of the studies about the effect of organic acids on the solubilization of phosphate minerals, the structure of the acids has generally been found more important than their quantity (Johnson,1959;Sperber,1957 and 1958; Swaby and Sperber, 1958; Struthers and Seiling, 1950). The following conclusions about the effect of structure of organic acids on P dissolution may be drawn from the literature available:

1. Amino acids have no significant effect on solubilization of phosphate minerals. Swenson *et al* (1949) found that amino acids were ineffective in complexing iron and aluminum at low pH, because the amino group acts as a hydrogen acceptor at low pH. At high pH, amino acids might become more effective. In soil,however,amino acids may be converted by soil bacteria to the corresponding hydroxy acids. These hydroxy acids effectively form complexes with iron and aluminum.

2. The ability of organic acids to prevent precipitation of phosphate is increased progressively as the number of functional hydroxyl groups increase. Johnston (1959) found that the hydroxyl derivatives were much more effective in solubilizing ferric phosphate than their unsubstituted parent acids. In this respect,he found that lactic and mandelic were more effective than propionic and acetic acids. Swaby and Sperber (1958) and Sperber (1957,1958) also found that a-hydroxy acids were more effective than volatile acids, presumably because they chelate with Ca, Fe or Al in addition to lowering the pH.
3. The number of carboxyl groups is an important factor in solubilizing phosphate minerals. In this respect tricarboxylic acids (citric) have the greatest effect, followed by dicarboxylic acids (malic) and then, monocarboxylic acids (lactic). Struthers and Sieling (1950) reported that malic acid was more effective in preventing precipitation of phosphate by iron and aluminum than was lactic acid.

In a recent study by Kwong and Huang (1980),it was shown that the non-crystalline state of aluminum hydroxides and the accompanying high specific surface area was maintained by precipitation in the presence of 10^{-4}M organic acid. This stabilized their high phosphate sorption capacities. On the other hand,the reversion of the non-crystalline precipitation products of Al to the

crystalline state at organic acid concentration of 10^{-4} M, drastically lowered their specific surfaces, with a concomitant decrease in the sorption of phosphate. Under these conditions, organic acids can reduce P concentration in solution.

Johnston (1954) examined the effect of aromatic acids on the solubilization of phosphate minerals. He found that the solubility of the aromatic acids in water did not significantly affect the extent of the solubilization of tricalcium phosphate by these acids. He also found the structure of the aromatic acid a very important factor in its ability to dissolve phosphate minerals. Mandelic acid solubilized the greatest amount,followed by salicylic acid. Benzoic acid solubilized the least amount of the tricalcium phosphate, but the substitution of one hydroxyl group into ortho and meta position increased the influence of the acid. From this, Johnston (1954) concluded that as the hydroxyl group became removed further from the carboxyl group of benzoic acid, the power to remove phosphate from tricalcium phosphate diminished.

Solubilization by sugar acids (such as 2-Ketogluconic acid) has been researched extensively. Mehltretter *et al* (1953) studied the ability of sugar acids to form complexes with metal ions. They found that the sequestering action of sugar acids was more pronounced in alkaline solution than in acid or neutral solutions. Presumably, this effect is due to the increase in dissociation of the hydrogen of hydroxyl

groups in sugar acids under alkaline conditions. Highly alkaline solutions,however,reduced the sequestering ability of sugar acids, possibly through the formation of stable sodium salts with the hydroxyl groups. They also observed that substitution of the keto group adjacent to the carboxyl group, appeared to enhance the sequestering power of a sugar acid. Thus, they found that 2 - ketogluconic acid has one third more sequestering power towards calcium than gluconic acid in 3% sodium hydroxide solution, and has 7 times the sequestering power of lactic acid.

Duff *et al* (1963), Duff and Webley (1959) and Louw and Webley (1959) explained the mechanism in which 2-ketogluconic acid dissolved phosphate minerals as one in which the acid forms a complex with calcium ions, leaving the phosphate ions in solution. Duff and Webley (1959) concluded that carbohydrate acids were effective chelating agents, and further , that the chelating ability of 2-Ketogluconic persisted even in acid growth media (pH 3 - 3.5). Also, they ascribed the decrease in pH to the chelation of calcium by the acid. The findings of Moghimi *et al* (1978) are in conflict with both Mehltretter *et al* (1953) and Duff and Webley (1959). Moghimi *et al* (1978) reported that above pH 5 the ability of K-2-gluconate to dissolve hydroxylapatite was not more than that of water, while below pH 5 this acid caused a greater dissolution. This observation made Moghimi *et al* (1978) suggest that either 2-Ketogluconic acid does not chelate calcium effectively, or

that 2-Ketogluconic acid chelates calcium but the hydroxylapatite is not dissolved by the chelation process. The buffering action of K-2-gluconate was greater than the water below pH 5. This suggested that the dissolution of hydroxylapatite by K-2-gluconate may be due to the buffering action of 2-Ketogluconate below pH 5 (Moghimi *et al* (1978). To investigate the above suggestions, Moghimi *et al* (1978) made the following determinations:

1. They compared the dissolution of hydroxylapatite with the calcium stability constants of different substances. They found that dissolution of hydroxylapatite increased with the calcium stability constant of the anion in the suspensions.
2. They measured the calcium stability constant for K-2-Ketogluconate in a range of solutions with different ratios of $CaCl_2$ / KCl at constant ionic strength (0.2). The results showed that,despite the increase of $CaCl_2$ from 0 to 66 mM in the solutions, the observed pH values during the titration were almost identical. They concluded that under the chosen conditions the stability constant for calcium- 2-Ketogluconate is negligible.
3. They measured the pKa for the acid and found it to be 2.66 in 0.2 M KCl at 25°C, which indicated that 2-Ketogluconic acid is one of the strongest mono-basic carboxylic acids.

From the above results Moghimi *et al* (1978) concluded that :

1. 2-Ketogluconic acid is a poor chelator for calcium.
2. The dissolution of hydroxylapatite was due to the buffering action of 2-Ketogluconic acid below pH 5.
3. The drop of pH accompanying the production of 2-Ketogluconic acid was a natural consequence of the low pKa of 2-Ketogluconic acid.
4. The erroneous conclusion of Duff and Webley (1959) was due to misinterpretation of chromotographic data.

The effect of organic acids on dissolution of phosphate minerals in buffered systems has also been studied. Sperber (1958), detected lactic, glycollic, citric and succinic acids after decomposition of carbohydrate, and investigated the effect of these acids upon the dissolution of apatite with or without calcium carbonate. The acids investigated were lactic, glycollic and citric. In the absence of calcium carbonate, all three acids solubilized phosphate. When calcium carbonate was present, the dissolution effect of all the acids was reduced, with the greatest reduction occurring with lactic acid. From these data, Sperber (1958) concluded that in calcareous soils, any organic acids produced would be neutralized by the free calcium carbonate, and dissolution of phosphate would be most unlikely. Struthers and Sieling (1950), observed that at any pH value, within the entire range of values for agricultural soil, there are some organic anions that are markedly effective in preventing precipitation of phosphate by iron and aluminum. The effectiveness of citric acid was greatest in the pH

range from 4 - 6, whereas that of lactic acid was greatest in the pH range from 4 - 9.

Increased phosphorus availability as a result of liming acid soils has been observed by many researchers (Beater, 1945; Dunn, 1943; Salter and Barnes, 1935). This may be due, in part, to the production of organic acids as a result of stimulating the activity of soil microorganisms.

Many researchers, however, have questioned whether organic acids are produced, or persist, in sufficient amounts in aerobic soils to appreciably effect phosphate solubility. According to Norman (1951), no accumulation occurred under aerobic conditions, as the majority of heterotrophic bacteria and fungi utilize most, if not all, the organic acids produced from carbohydrate or protein oxidation. Stevenson (1977) reported that, as a result of the decomposition of organic residue by saprophytic organisms, organic acids and/or humic acids may accumulate in sufficient amounts in localised zones to appreciably increase the local or microsite availability of phosphate without affecting the overall phosphate solubility of the soil.

2.2.2 Effect of Carbon Dioxide.

Carbon dioxide produced by microorganisms and plant roots can lower the pH around them, possibly causing dissolution of phosphate minerals. Stoklasa and Ernest (1909) and Aberson (1910) as cited by Parker (1924) ,

studied the assimiliation of phosphorus from soil culture fertilized with different phosphates, and saturated three times a week with CO_2. They found that the CO_2 treatment was favourable when used with soluble phosphates, but with insoluble phosphates, it was not beneficial. On the other hand, Parker (1924) and Johnston and Olsen (1972) reported that CO_2 had no effect on P uptake by plants. When CO_2 was removed from the nutrient solution of growing wheat, barley, soybean and squash, no significant effect on dissolution of fluorapatite or the quantity of P absorbed by plants occurred (Johnston and Olsen. 1972).

2.2.3 Effect of Sulfur Oxidation.

About one hundred years ago, Charles F. Panknin (1877), as cited by Lipman *et al* (1916), reported that when a mixture of ground bone or ground mineral phosphate and sulfur was added to soil, sulfuric acid was produced by a natural oxidation of sulfur, which then dissolved ground bone and mineral phosphate making phosphorus more available. Lipman *et al* (1916) noticed that the oxidation of sulfur in inoculated soil far exceeded that of sterilized soils. From this observation, they concluded that the oxidation of sulfur was largely biological, and the presence of bacteria, essential. In 1904, William B. Chisholm, as cited by Lipman (1916), also found that when a mixture of rock phosphate and sulfur was ground to powder and applied to soil, crop production was apparently far superior to what might

reasonably be expected from the amount of available phosphorus, nitrogen, potassium and other minerals present in the fine mixture.

Recently, Swaby (1974) (cited by Bromfield,1975),Swaby (1975 and 1976) (cited by Cosgrove, 1977), and Bromfield (1975) studied the use of biosuper (rock phosphate - sulfur mixture). They found that a rock phosphate - gypsum mixture was consistently inferior to biosuper, both in crop dry matter production and P and S uptake by plants. The increase in yield, as a result of the addition of biosuper, might alternatively, be due to an increase in the availability of micronutrients by the action of the acid on soil minerals (Hassan and Olsen, 1966), or as a result of a better root system produced by additional sulfur supply (Bromfield,1975).

Many factors would affect the oxidation of sulfur, and consequently affect the solubilization of rock phosphate. These factors are :

1. Organic Matter.

   Vermorel and Dautony,(1913), and Brown, as cited by Lipman *et al* (1916), found an increase in sulfur oxidation when organic matter was added to the soil, but Brionx and Guerbet (1913), as cited by Lipman *et al* (1916), found that carbohydrates had a retarding effect, while peptone and certain other nitrogenous materials accelerated sulfur oxidation.

2. Moisture and Temperature.

Between 40% and 50% of saturation were found to provide optimum moisture conditions for S oxidation (Lipman *et al* ,1916) Swaby and Fedel,(1977) found that sulfur oxidation was more rapid in the humid tropics than in temperate or arid regions. They ascribed this to the lower activities of thiobacilli in temperate and arid regions. They concluded also, that many tropical Australian soils lack sufficient sulfur bacteria to oxidize sulfur rapidly. These soils could benefit from inoculation with thiobacilli, but the initial rate of release of soluble phosphate is still too low to satisfy the requirements of the crop at the important early stages of growth (even in wetter tropical regions) (Swaby, 1976, as cited by Cosgrove (1977).

3. The Ratio of S:P_2O_5 in the Mixture.

The S:P_2O_5 ratio,in biosuper,is an important factor affecting release of phosphate from rock phosphate. Swaby, (1976) produced pellets containing 16.6 % S and 18.5 to 26.1 % P_2O_5 and a soil inoculum of thiobacilli (0.1 %). He claimed that this could be used economically in the wetter tropics, particularly, where the annual rainfall is greater than 635mm. In temperate regions, this preparation is less successful, owing to the lower rate of thiobacilli activity. Lipman *et al* (1916) suggested the ratio 9:15 would satisfy both the sulfur and phosphorus requirements of the crop. Bromfield, (1975) suggested that the ratio S:P_2O_5must be adjusted

periodically because of differential build up of P and S in soil.

The use of biosuper is an attractive possibility, particulary in countries where the cost of the production of superphosphate is high and the soil temperature and moisture regimes are satisfactory.

2.2.4 Effect of the Microbial Population.

The abundance of microorganisms in the rhizosphere of plants is much greater than in non-rhizosphere soil. Organic substances excreted by roots,such as carbohydrates, as well as organic material from dead cells, are easily attacked by most microorganisms. Harmsen and Jager (1963) found that roots excreted from 120 - 1000 ppm carbon into the soil immediately adjacent to the roots; 120 - 800 ppm in the surrounding 1 cm thick layer and 0 - 40 ppm in the more distant remaining soil. The organic materials excreted by the roots are the primary cause of microbiological activity in the rhizosphere. Thus, in the rhizosphere, special processes may take place, which are of utmost importance to the uptake of nutrients by plants. Hayman (1975) reported that root exudates and sloughed cells provide substrate to support the intense microbial activity characteristic of the rhizosphere. Rovira (1956), showed that the essential energy materials used by microorganisms include amino acids and sugars exudated from roots.

Gerretsen (1948) was the first to draw attention to the role of the rhizosphere microflora in P nutrition. He found that plants grown under sterile conditions were unable to use apatite, whereas in non- sterile conditions, insoluble phosphate apparently became available. Swaby and Sperber (1958) confirmed the findings of Gerretsen (1948) and reportd that under sterile conditions plant growth was inferior to that under non sterile conditions. The numbers of phosphate dissolving organisms in the rhizosphere of plants generally exceed those in the non-rhizosphere soil. In most cases the proportion of these microorganisms in the rhizosphere population is much higher than in the control soil. Sperber (1957 and 1958) found that 20 - 40% of the organisms isolated from rhizospheres of different plants dissolved hydroxylapatite, whereas only 10 - 15% of those isolated from control soil dissolved this compound. Katznelson and Bose (1959) observed approximately 6 times as many phosphate dissolving bacteria in rhizosphere soil, and about 18 times as many in the rhizoplane as in control soil. They found no difference, however, in the proportions of phosphate dissolving bacteria in the rhizoplane, rhizosphere and root free soil. Bacteria on seeds were also studied by Katznelson *et al* (1962) who found that 40 - 70 % of them dissolved phosphate.

The presence of a large population of phosphate dissolving organisms in the rhizosphere, combined with available energy sources in the form of root excretions,

suggest that the root zone is a likely site of P solubilization in soil. Therefore, solubilization of minerals and the uptake of phosphorus by plants must be considered within the rhizosphere environment. Beyond this region, which coincides with the zone of phosphate depletion, solubilization would have little effect, because of the slow diffusion of phosphate ions in soil. This zone of depletion is about 1 -2 mm around the roots, which is also the region where microorganisms are particularly active. (Hayman, 1975).

The presence of calcium carbonate and other buffering material would, however, rapidly neutralize organic or inorganic acids, which appear to be the primary cause of solubilization. Swaby and Sperber (1958) listed the following as conditions in which microorganisms may appreciably increase the availability of insoluble phosphate. :

1. In sandy soils, either naturally or artificially acidic, where extra production of organic acids by microorganisms enables acid tolerant plants, such as lupins and seradella to thrive.
2. In old pastures where rhizosphere microorganisms and humic acids are very plentiful, so that lucerne and white clover benefit from the solubilized phosphate.
3. In light soils, low in sesquioxides, high in organic matter and sulphates, and subject to water-logging, where organic acids and sulphides may increase phosphate

solubilization.

Sperber (1958), however, claimed that in soil there is no lack of organisms, but rather, there is a need for substrates to stimulate these organisms to produce sufficient phosphate dissolving products.

Methods of isolation or detection of phosphate dissolving microorganisms, and methods for measuring the capacity of these microorganisms to dissolve different phosphate minerals have been studied for the past 70 years. Most of that work was carried out with pure cultures under optimum temperatures using laboratory media containing sufficient substrate, no antagonism from other microbes, no absorption by clay minerals and little buffering. But in soil the situation is different. There is always competition between soil organisms for substrates together with interactions between organisms, soil particles and soluble phosphate.

Sucket *et al*,(1908), as cited by Louw and Webley, (1959), were the first to detect phosphate dissolving microorganisms using plating methods. They used tricalcium phosphate and dicalcium phosphate as the only source of phosphate, and used clear zones around colonies to indicate the ability of these micoorganisms to dissolve phosphate. In 1919,Fred and Haas, as cited by Gerretsen (1948) showed that the etching of marble by the roots of peas was increased in the presence of bacteria. Gerretsen, (1948), concluded that microbes in the rhizosphere played an important role in this

phenomenon. Sperber (1957; 1958) also used this method and observed "digestion" zones varying from 1 to 10 mm in diameter around individual colonies. Plating techniques were not, however, able to detect microorganisms capable of dissolving gafsa rock phosphate, and no clear zones were observed around the colonies (Louw and Webley, 1958; 1959). When an analytical method was used, 82 % of these microorganisms were able to release phosphate from gafsa rock phosphate.

Gerretsen (1948) isolated phosphate dissolving bacteria from rhizospheres using precipitated phosphate in nutrient-agar prepared by adding 15 ml of sterilized 10 % $CaCl_2$ solution and 10 ml of sterilized 10 % K_2HPO_4 solution to the medium after autoclaving. He laid the roots and adhering particles onto the nutrient agar medium containing the precipitated phosphate and observed two opposite cases. The first was dissolution of phosphate as indicated by clear zones around the colonies. The second was precipitation of phosphate. These two cases were also observed by Sperber (1957; 1958). Whether solubilization or precipitation occurs may depend on whether acids are produced from sugars or alkalis from nitrogen - containing compounds (Gerretsen, 1948).

The capacity of phosphate dissolving microorganisms to dissolve P has generally been measured using liquid media with the amount of orthophosphate in solution as the definitive parameter. The main acid produced by these

bacteria was lactic acid (Sperber, 1957). Louw and Webley (1959) found that more than 100 isolates from the root region of oat plants produced haloes around their colonies on a dilution plate containing calcium carbonate or dicalcium phosphate, and 21 out of 26 of the isolates tested produced mainly lactic acid.

Katznelson and Bose (1959) found that a high percentage of phosphate dissolving bacteria required yeast extract and soil extract for optimum growth. Lochhead and Chase (1943) have pointed out that these bacteria are predominantly pleomorphic forms such as soil *Corynebacteria* and *Arthrobacter*. The genus *Arthrobacter* has been found by many researchers to be effective at dissolving insoluble phosphate (Sperber, 1958; Swaby and Sperber, 1958; Katznelson and Bose, 1959).

Although phosphate dissolving bacteria have been extensively studied , much less attention has been devoted to fungi. In this respect, Rose (1957) showed that some fungi could dissolve ferric phosphate. *Aspergillus ferreus* (apparently through production of H_2S) brought a greater amount of ferric phosphate into solution than any other organism tested. He also found that *Aspergillus niger*, *A.ferreus* and *Sclerotinum rolfsii* produced oxalic and citric acids and effectively dissolved insoluble phosphate.

Chhonkar and Sabba - Rao (1967) detected the solubilization of insoluble tricalcium phosphate by fungi associated with root nodules of different legumes. He found

that *Aspergillus niger* lowered the pH to 2.9 and solubilized tricalcium phosphate, both in the presence and absence of soluble phosphate (KH_2PO_4). Other isolates such as *Penicillium lilacinum*, *A.flaws*, *A. nidulans* and *A. terrens* solubilized tricalium phosphate in the absence of soluble phosphorus, but failed to do so in the presence of soluble phosphorus which they utilized as a P source. Agnihotri (1970) recognised three groups of phosphate dissolving fungi :

1. those which reduced the pH, such as *Sclerotium rolfsii* and *Aspergillus niger*
2. fungi which increased the pH of the medium, such as *Rhizoctonia praticola*
3. fungi which did not appreciably alter the pH.

Species of the genera *Penicillium*, *Aspergillus* and *Rhizopus* have been isolated from seeds of several plants and found capable of dissolving insoluble phosphate minerals.

Sperber (1958a) concluded that plants exert a selective effect on phosphate-dissolving bacteria, because he found that most of the isolates rapidly and irreversibly lost their ability to dissolve phosphate after a few subcultures, although alternate interpretations of such observations are warranted. Among the plants he used were subterranean clover, wimmera ryegrass, perennial ryegrass and wheat. Webley and Duff (1965) found that the proportion of phosphate dissolving bacteria which produced 2-Ketogluconic acid were 1.3%, 1.2% and 0.03 % for the root surface,

rhizosphere soil and control soil of barley respectively. These data suggested that barley may exert a selective effect on P dissolving bacteria. In contrast, Katznelson and Bose (1959) observed no selective effect of wheat roots on microorganisms which dissolved $CaHPO_4$. They suggested that because of the greater metabolic activity of rhizosphere and rhizoplane organisms, it is quite conceivable that a greater phosphate turnover occurs at the root-soil interface. Also, no general preferential stimulation of phosphate dissolving bacteria by roots was found by Katznelson *et al* (1962). They showed that barley appeared to exert a favourable effect on those bacteria, whereas the reverse occurs with oats. For corn, red clover and flax, the percentage occurrence of phosphate dissolving bacteria in the rhizosphere or rhizoplane was not different from that in the non-rhizosphere soil.

Inoculation of plants with bacterial fertilizers has been suggested as a way to obtain yield increases in the field. The inoculation of plants or seeds with *Azotobacter spp.*, *Bacillus megatherium*, var. *phosphaticum* or *Rhizobium spp.* is referred to (in the U.S.S.R.) as azotobacterin, phosphobacterin and nitragin respectively.

Inoculation of plants with phosphobacterin is still a subject of controversy. Scientists in the Soviet Union claim that phosphobacterin increases crop yields. Kudachev (1956) reported that inoculation of seeds with liquid phosphobacterin at a rate of 50 ml ha^{-1} increased yields and

protein content in grains. Increasing the rate from 50 to 100 ml ha^{-1} gave further increase in yields of grain and total yield of protein. Mishustin and Naumova (1962) reported that plants inoculated with azotobacterin and phosphobacterin showed better germination, growth and yield. On irrigated chestnut and grey soil, bacterial fertilizers increased growth of both shoots and roots of lucerne and cocksfoot. The most effective was phosphobacterin (Zenkova, 1955). Zenkova (1955) also observed that a mixture of two kinds of bacterial fertilizer gave better results than when they were applied separately. Because field experiments in the Soviet Union are rarely designed for statistical analysis, it is not easy to evaluate their data (Smith *et al* (1961).

Smith *et al* (1961) designed a greenhouse experiment to evaluate the effect of phosphobacterin. They found that the yield of tomato was increased by 7.5 %, which was significant at the 1% level. With wheat, no statistically significant differences were attributed to the added inoculum, although they ran their experiments with Chernozem and Chernozem-like soils that had been reported by the Soviet scientists to be their most responsive type.

The discrepancy between the results found in the Soviet Union and those found by Smith *et al* (1961), may be explained by the following :

1. Differences in cropping systems and soils. But this alone is not enough to explain the differences. (Smith

et al, 1961).

2. In the Soviet Union a systematic statistical approach is little used. This may explain part of the problem (Cooper, 1959).
3. The most likely explanation is that phosphate-solubilizing bacteria are common in most soils, and the addition of a few more organisms, such as *B. magatherium*, would not always be expected to produce significant yield increases.

2.3 Mycorrhiza.

2.3.1 Introduction.

Mycorrhiza is a unique fungus associated with higher plants. The ecological niche of the fungus is properly within the root association.

The formation of mycorrhizae is favoured by land low in phosphorus and nitrogen. Daft and Nicholson, (1965) reported that , in general, it was difficult to obtain growth increases with mycorrhizal infection where the supply of readily available nutrients was high. Plants without mychorrizae are not often found in natural or cultivated soil. Such plants, however, generally take up much less phosphorus from soil deficient in labile phosphate than do plants with mycorrhizae.

Mycorrhiza can be divided into two groups :

1. Ectomycorrhiza, in which the fungus forms a mantle around the exterior of the roots consisting of a network composed of a mass of hyphae entering into spaces between the individual plant cells.
2. Endmycorrhiza, in which the fungus penetrates the cells of the host plant.

2.3.2 Ectomycorrhiza and Phosphorus Uptake.

Morphological changes in roots induced by mycorrhizal infection can increase uptake by enabling them to explore more soil. The loose hyphae extending out from the sheath exploit soil beyond the P - depletion zone, and ramify in the phosphate - rich humus, thereby increasing the area of absorbing surface in contact with the phosphate pool (Hayman,1975). Stone, (1949) found an extensive development of external mycelium associated with seedling uptake of phosphate. The P was believed to have entered through the fungal mycelium, rather than from labile phosphate released into the soil by the fungus. He also showed that the mycorrhizal fungi depleted rather than contributed to the labile pool.When Sudan grass was grown together with mycorrhizal pine, it absorbed very little phosphorus and grew very poorly, but it absorbed between 3 and 10 times as much phosphorus when the pine was not mycorrhizal. The non - mycorrhizal pine absorbed little phosphorus. These data demonstrate the effect of mycorrhizae on the competition

between the plants for P.

Bowen and Rovira,(1969), as cited by Hayman (1975), found that mycorrhizal roots function longer than non - mycorrhizal roots, thereby continuing to use the small amounts of phosphate diffusing very slowly into P - depletion zones.

2.3.3 Endomycorrhiza and phosphorus uptake.

It is generally accepted that large growth responses can be attributed to development of vesicular-arbuscular (VA) mycorrhiza on the root system of plants growing in phosphorus deficient soils. These growth responses are usually associated with improved phosphorus nutrition of the host. Unlike the ectomycorrhizae, VA mycelia cause little change in root morphology.

In many low-phosphate soils, inoculation of plants with *Endogone spp.* has increased phosphate uptake several fold, an effect comparable to adding monocalcium phosphate (Hayman and Mosse, 1971, and Mosse, 1973). Hall,(1978), found higher concentrations of P in infected plants than in non-mycorrhizal plants. Hayman (1975) suggested that mycorrhizal plants could use forms of soil phosphorus that non-mycorrhizal plants could not use, however this conclusion is not generally held.Daft and Nicholson (1966) and Murdoch *et al* (1967) found that plants benefited from VA mycorrhiza in the presence of insoluble phosphates, such as bone meal or rock phosphate. This suggested the

solubilization of these materials by the VA fungi. To test this hypothesis, Hayman and Mosse (1971) used ^{32}P and found that, although the mycorrhizal plant took up more phosphorus than non-mycorrhizal plants, the ^{32}P / ^{31}P ratio in the mycorrhizal and non-mycorrhizal plants was the same and matched that in solution. This indicated that mycorrhizal and non-mycorrhizal plants had used the same labile source of phosphate. In contrast Mosse *et al* (1973) found non-mycorrhizal plant contained no ^{32}P, while mycorrhizal plants contained ^{32}P activity and grew much better. Mosse *et al* (1973) explained this discrepancy on the basis that non-mycorrhizal roots of some species cannot utilize P present at very low concentrations in the soil solution but mycorrhizal roots can. Thus, the mycorrhizal effect is not due to the use of unavailable sources of P, but simply to more efficient use of the available forms (Gerdemann, 1975). Ross and Gillian (1973) found that plants utilized the most readily available P irrespective of whether they have mycorrhizae or not.

Hattingh *et al* (1973) found that VA mycorrhizal hyphae could transport ^{32}P across at least 15 mm of soil into an infected root. Rhodes and Gerdemann, (1975) in their studies with ^{32}P labelled soil, have shown that mycorrhizal roots were able to acquire ^{32}P placed 8 cm from the root,while non-mycorrhizal roots failed to take up ^{32}P placed 1 cm from the root. Fungicides added 48 hours before ^{32}P addition reduced ^{32}P uptake of the infected plant to the level seen

in mycorrhizal plants (Gray and Gerdemann,1969). This was direct evidence that a mycelial network around VA mycorrhizal roots can extend the region of phosphate removal well beyond the P-depletion zone. Bieleski (1973), as cited by Hayman (1975), reported that 1 mm of mycorrhizal root connected to soil by four hyphae, each one 25 um in diameter and 20 mm long, would have a P uptake 60 times greater than the same length of uninfected root,if diffusion was limiting, and 10 times greater if uptake were proportional to the surface area. Sanders and Tinker (1973) concluded that the increased efficiency of P uptake by mycorrhizal roots could be accounted for by the uptake and transport of P by the mycorrhizal hyphae themselves.

Hyphal strands growing out into the soil from mycorrhizal roots are somewhat analogous to root hairs. The lack of root hairs in some plants could explain their dependence on mycorrhizae for P uptake unless given soluble phosphate (Baylis, 1970). Other plants with poor root hair development, such as onions and citrus, also benefit from VA mycorrhiza. Nevertheless, Mosse *et al* (1973) found that some plants with extensive root hair development benefit from VA mycorrhizae. This suggests that more is involved than just the physical effect of the hyphae exploiting more soil.

Kucey and Paul (1980) listed four mechanisms by which the fungus could enable the plant to absorb more P :

1. Alteration of plant hormones such that the plant produces a larger root system.

2. Absorption of P from sources unavailable to the plant, i.e. rock phosphate or organic P.
3. By producing or causing the plant to produce more efficient enzymes for absorbing P.
4. Acting as a secondary root system, thereby extending the P depletion zone and increasing absorptive surface area.

Accelerated depletion of soluble phosphate by mycorrhizal plants is not of long-term benefit in agriculture. Both mycorrhizal and non- mycorrhizal plants are less able to extract P from the soil as it becomes increasingly more depleted (Powell,1977). There is, therefore, a need to continue studying both aspects of mycorrhizal stimulation of the bacterial population in the rhizosphere, and the possibility that PDB and mycorrhizae inoculated separately or together might enhance the ability of plants to use P from sparingly soluble phosphate minerals.

2.3.4 Bacteria-Mycorrhiza Interaction.

Strains of mycorrhizal fungi for inoculation should be selected, not only on their ability to stimulate plant growth in soil under ideal conditions, but also on their ability to establish and persist under conditions encountered in the field, and on their ability to function in a mixed biological system. To this end, Bowen and Theodorou (1979) studied the interaction between ectomycorrhizae and soil bacteria and observed the following

:

1. Depression of mycorrhizal growth by some bacteria, e.g. *Bacillus sp* and *Pseudomonas sp*. This may be due to:

    a. Competition for energy source. Bowen and Theodorou, (1979) observed that the addition of glucose greatly minimized the reduction of mycorrhizal growth.

    b. Antibiosis: This effect was clear with *Pseudomonas fluorescence* , as the addition of glucose to the soil did not increase growth of *Pseudomonas* but, instead, increased suppression of *Rhizopogon luteolus*.

2. Stimulation of some fungal growth by bacteria.

    They found that *Bacillus spp* stimulated ectomycorrhizal fungi. This might be due to increased leakage of substances from the root in the presence of bacteria (Bowen and Theodorou, 1979).

3. In some cases no effect was observed, e.g. *Bacillus sp* 3H19 and *Bacillus sp* S80. From these results, Bowen and Theodorou suggested the following:

    a. The effect of bacteria depends on the combination of organisms.

    b. The interaction between bacteria and mycorrhizae should be studied in the rhizosphere rather than in laboratory media, because the interaction is affected by the environment.

    c. Mycorrhizal fungi for inoculation must be

selected on the basis of compatibility with the microflora in a wide range of soils, as well as on their potential for increasing plant growth in controlled experiments in sterilised soils in the greenhouse.

Unlike mycorrhizal fungi, which can become well established inside the plant root, phosphate dissolving bacteria must establish themselves in the rhizosphere in competition with many other organisms. Bacteria introduced into the rhizosphere as seed inoculum generally did not maintain a high population and their number dropped to sub - optimal levels (Barea *et al*,1975). Raj *et al* (1981), Azcon *et al* (1976) and Barea *et al* (1975) showed that *Endogone* enabled phosphate dissolving bacteria introduced into the rhizosphere to maintain higher numbers for longer periods than in the absence of mycorrhizae. This effect may prolong the metabolic activities of these bacteria in the rhizosphere, thus increasing their opportunity to influence plant growth. Further, hyphae of *Endogone* may absorb and translocate into the plant any soluble phosphate ions that the bacteria released from insoluble phosphate. Azcon *et al*,(1975) noticed that uninoculated plants derived no benefit from rock phosphate added to the soil. Inoculation of four-week-old seedlings of lavender with *Endogone E*, and phosphate dissolving bacteria, significantly increased plant growth above that achieved with either organism separately (Azcon *et al* 1976). The combined inoculation of *Endogone* YV

(yellow vacuolate) and phosphate dissolving bacteria, however, did not significantly increase plant growth above YV alone. YV-inoculated plants grew best of all, regardless of whether bacteria or rock phosphate were added. Nevertheless, plants inoculated with YV and phosphate dissolving bacteria took up most phosphate (Azcon *et al*, 1976). This suggested that some phosphate entered the soil solution, either from solubilization of rock phosphate by the bacteria, or from mineralization of dead bacteria, because more bacteria grew and died around the mycorrhizal than the non-mycorrhizal plants (Azcon *et al*, 1976). Raj *et al*, (1981) observed that inoculation of sudan grass with VA and phosphate dissolving bacteria, either separately or combined, increased phosphate uptake, as well as dry matter yield of plants. No differences were observed, however, in the ^{32}P / ^{31}P ratio between the plant and soil solution after adding ^{32}P labelled tricalcium phosphate. This indicated that both absorbed P from the same pool.

2.4 The Role of Dissolution of Phosphate Minerals in Plant Nutrition.

Plants grown in soil inoculated with phosphate dissolving bacteria absorb more phosphate and grow better than those grown in sterilized soils (Gerretsen, 1948; Brown, 1974; Barea *et al*, 1976; Azcon *et al*, 1978).

Gerretsen, (1948) studied the effect of microorganisms in soil on uptake of phosphorus by rye,mustard and oats. In all his experiments, he found that inoculating quartz sand with 1 % garden soil and adding insoluble phosphate minerals, increased both the amount of phosphorus absorbed and the dry weight of the plant (Table 1).

Whether or not the stimulation of plant growth and increased P uptake occurs due to solubilization of insoluble phosphate minerals is still a matter of controversy. Brown, (1974), Barea *et al* ,(1976) and Azcon *et al*,(1978) interpreted the effects on plant growth that resulted from inoculation with phosphate dissolving bacteria as being caused primarily by growth regulators. Increased root growth (Barea *et al*,1976) could account for the increase of uptake of P. Phosphate dissolving bacteria may then play a secondary, or indirect, role in bringing extra soluble P into soil solution from sparingly soluble sources, especially in P-deficient soils (Barea *et al* , 1976). Azcon *et al* (1978) suggested that plant growth substances produced in the rhizosphere by phosphate dissolving bacteria could affect the early stages of plant growth (Brown,1974), so that actively growing roots were able to explore more soil and more zones where phosphate ions were chemically dissociated from rock phosphate particles. This hypothesis was supported by the work of Johnston and Olsen (1972) who found that close proximity of apatite particles to plant roots was essential for their dissolution.

Table 1: Yield and phosphate absorbed by Oat Plants grown for 70 days in sterile and infected quartz sand. (From Gerretsen, 1948)

Treatment	Total dry wt. of 5 Plants (mg)	Increase in yield by infection (% of Sterile)	Total P_2O_5 (mg) in the plants	Increased P_2O_5 in plants compared with Sterile (%)
Blank, without phosphate	2.0	-	1.8	-
Ferrophosphat (S)	2.5	72	6.3	120
" " (In)	4.3		13.9	
Algeria phosphate (S)	2.5	92	2.6	324
" " (In)	4.8		11.0	
$CaHPO_4$ (S)	3.1	187	28.5	124
" (In)	8.9		66.0	
$Ca_3(PO_4)_2$ (S)	3.4	188	36.4	120
" (In)	9.8		80.1	
Bone Meal (S)	5.2	138	42.0	79
" (In)	12.4		75.2	

(S) = Sterilized
(In) = Infected

Sundara Rao *et al* (1963) examined the effect of *Bacillus megatherium* (phosphobacterin) on 0.05 N HCl extractable phosphorus and on P uptake by wheat from native and added ^{32}P phosphate minerals. When bonemeal, rock phosphate, superphosphate and hydroxylapatite were added to Karnal soil (pH 6.8), percentages of the respective phosphates solubilized were: 24, 18, 22 and 41. The percentage of ^{32}P released from tagged superphosphate and tagged hydroxylapatite were 28.57 and 38.17 respectively.About one third of the phosphorus absorbed by wheat was from the added ^{32}P insoluble phosphate mineral. These results suggested that the solubilization of phosphate minerals by phosphobacterin played an important role in bringing more phosphate into soil solution (Sundara Rao *et al*, 1963).

Raj *et al*, (1981) observed higher extractable ^{32}P (30 mM NH_4F-HCl) and greater P uptake from soil when finger millet (*Eleusine coracava*) seedlings were inoculated with *Bacillus circulans*, a phosphate dissolving species. This species did not produce growth-promoting substances, such as IAA or GA_3, in pure cultures and there was no evidence of enhanced root growth due to bacterial inoculation. Azcon *et al*,(1978),however, reported that cell free supernatants of certain phosphate dissolving bacteria promoted plant growth in a manner similar to applied plant hormones. Obviously, inoculants that produce several growth promoting substances and are active solubilizers of phosphate minerals would be

likely to give the most beneficial effect. (Barea *et al*, 1976).

Besides hormonal effects and the effect of solubilization of phosphate minerals on plant growth, Brown (1974) suggested other important factors. These factors are :

1. Changes in the rhizosphere microbial population.

   It was found (Brezova, 1963; Meerediak, 1964; Mosiashvili *et al*,1963 and Samtsevich, 1962, as cited by Brown, 1974) that inoculation of plants with *Azotobacter* and *Bacillus megatherium* increased the activity of rhizosphere bacteria involved in ammonification, N_2-fixation, nitrification, phosphate mineralization and cellulose decomposition.

2. Disease suppression by inoculants.

   Dorosinski, (1962); and Samtsevich, (1962); as cited by Brown (1974) reported that inoculation with *Azotobacter* and phosphobacterin (*Bacillus megatherium* decreased occurrence of viral and bacterial disease of potato. Brown (1974) found wheat infected with *Gaeumannomyces graminis* var. *titrici* grew better when inoculated with *Azotobacter* , but take-all disease was not decreased.

Cultures of *Bacillus* and *Pseudomonas* frequently inhibit plant pathogens when tested *in vitro*, and might give disease control as inoculants . For example Kleopper *et al* (1980) found that inoculation of plants and seeds of potato, radish

and sugar beet with specific strains of *Pseudomonas fluorescens-putida* caused yield increases up to 144 % in field tests. They ascribed the increase in yield to the production of siderophores by *Pseudomonas* strains. These siderosphores bound iron effectively and made it unavailable to plant pathogens such as *Erwinia carotovora*

The following points about the role of microflora in the solubilization of sparingly soluble phosphate minerals and plant nutrition may be concluded from the above discussion:

1. The role of microflora in P nutrition is indisputable. Whether or not the stimulation of plant growth by phosphate dissolving microorganisms occurs due to solubilization of sparingly soluble phosphate minerals is still a subject of controversy.
2. Bacteria and fungi capable of dissolving sparingly soluble phosphate minerals have been isolated from soil, and from the rhizosphere and rhizoplane of several plants.
3. Solubilization of sparingly soluble phosphate minerals by PDM may be caused by one or more of the following mechanisms.

    a.Lowering the pH with organic or inorganic acids produced in soil as a result of microbial activity.

    b.Chelation of calcium, iron and aluminum by organic acids.

    c.Formation of phosphohumic complexes.

d. Production of H_2S which reacts with ferric phosphate precipitating iron as ferrous sulphide and liberating phosphate.

e. Competition between humate and phosphate ions for absorbing surfaces.

4. In order to be beneficial to the plants, solubilization of phosphate minerals by PDM must be considered within the rhizosphere environment.
5. Inoculation of seeds or plants with phosphate dissolving bacteria (PDB) is still a subject of controversy. Scientists in the Soviet Union claim that inoculation of seeds with PDB has a beneficial effect, while scientists in the United States have found few significant increases in yield due to inoculation with PDB.
6. The only role of PDB that has been stressed in the past is the increase of P concentration in solution. Pumping P from phosphate minerals into labile organic forms has been ignored.

3. Methods of Analysis

3.1 Measurement of Phosphorus.

3.1.1 Soluble Inorganic P (Psi)

Psi was measured using the molybdenum blue method of McKeague (1978).

3.1.2 Soluble Organic Phosphorus (Pso)

Pso was measured indirectly as the difference between total soluble phosphorus and Psi following organic P digestion.

Digestion of Pso:

Sample solutions (25 ml) were transferred into 50ml erlenmeyer flasks and dried at 70°C. A digestion solution was prepared by mixing 14 g of lithium sulphate ($Li_2SO_4.2H_2O$), and 350ml of H_2O_2, into 420ml of H_2SO_4 according to the method of Parkinson and Allen (1975). Selenium was omitted because it was found to interfere in the determination of phosphorus when the molybdenum blue method was used. Five ml of the digestion solution were added to each flask and the contents were digested on a hot plate at 140°C until they dried. This was necessary to guarantee the reduction of H_2O_2 which would interfere by oxidising ascorbic acid, which is used to reduce molybdate complexes to produce the final blue colour. The dry contents

were dissolved in deionized water and quantitatively transferred into a 100ml volumetric flask. The total P was determined using the molybdenum blue method and the soluble organic phosphorus was calculated as follows:

Pso = Total Soluble P - Psi

3.1.3 Phosphorus in Bacterial Cells

Following separation of cells from the culture solution and from hydroxylapatite, they were digested using the method previously described. Phosphorus was then determined using the molybdenum blue method. The method of separating cells from hydroxylapatite formed a significant component of this study and its development is treated in a later section.

3.2 Measurement of Calcium

Calcium was measured in the solution using a Perkin-Elmer Atomic Absorption Spectrophotometer Model 503. All instrument settings and procedures followed are those published in the analytical procedures manual for the Model 503. Lanthanum was added to the sample as well as to the standard to bring the final concentration of Lanthanum in the sample to 2000ppm (Pawluk, 1967).Lanthanum was necesary to prevent interference of P.

3.3 Measurement of pH

pH was measured using an E488 Metrohm Herisau pH meter with a combination electrode.

4. Isolation and Qualitative Studies.

4.1 Isolation of Phosphate Dissolving Bacteria (PDB)

The agar plate method (APM) with precipitated phosphate in the medium (Gerretsen, 1948) was used to isolate phosphate dissolving bacteria from soil and to test pure cultures for their ability to dissolve precipitated phosphates in agar media.

In this method a medium composed of

0.25g $(NH_4)_2SO_4$

0.05g $MgSO_4.7H_2O$

0.10g $MgCl_2 H_2O$

0.005g $FeCl_3$

0.50g Asparagine

0.50g Yeast Extract

5.0g Glucose

7.5g Agar

500 ml distilled water

was prepared and autoclaved at 120°C and 103 kPa for 40 minutes . Insoluble phosphate was precipitated in the medium by the adding of 15 ml of sterilized (by autoclaving) 10% K_2HPO_4 solution and 25 ml of sterilized (by autoclaving) 10% $CaCl_2$ solution to each 500 ml of the medium after the medium had cooled to 65°C. The medium was then aseptically adjusted to pH 7.0 using predetermined amounts of sterilized 0.5 N NaOH solution, and the plates were poured immediately.

Several dilutions, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7}, of a soil sample from the Ah horizon of a Black Chernozemic soil were prepared and the plates were inoculated and incubated at 25 °C for 14 days. Clear zones around the colonies were taken as indicating an ability to dissolve the precipitated phosphate in the media. Such colonies were picked and further purified by streaking onto precipitated phosphate medium. Seven isolates from soil had the ability to dissolve the precipitated phosphate using these critera. They were given the code numbers S_1 - S_7. Unfortunately all of them died before the complete identification was finished. Some characteristics of these isolates, however, are shown below.

1. Culture S_1 = Gram positive,cocci,facultative,not fluorescent, produces catalase and urease, does not use arabinose and raffinose as a carbon source but uses glucose and produces acids without gas, colonies are yellow, moist, round and medium. Probably *Streptococcus sp.*
2. Culture S_2: Gram positive, cocci, facultative, not fluorescent, does not produce catalase or urease, does not use arabinose as sole source of carbon and produces acids without gas, colonies are white, moist, irregular and smooth. Probably *Streptococcus sp.*
3. S_3: Aerobic,not fluorescent, produces catalase and urease, does not use arabinose, lactose or raffinose as sole source of carbon, oxidizes glucose, grows in clumps in broth culture , colonies are cream, round and dry.

4. Culture S_4: Aerobic,not fluorescent, produces catalase and urease,does not use arabinose, lactose or raffinose as sole source of carbon, oxidizes glucose, colonies are yellow, moist, round and small.
5. Culture S_5: Gram variable, rods, terminal spores, the rods have a slight curve at one end,does not use lactose as sole source of carbon, oxidizes glucose, colonies are large, white, irregular and smooth.
6. Culture S_6: Facultative, not fluorescent, produces catalase and urease, does not use arabinose or raffinose, oxidizes glucose and lactose and produces acids, colonies are white, small and moist.
7. Culture S_7:Gram positive, cocci, facultative, not fluorescent, does not produce catalase or urease, does not use arabinose as sole source of carbon, uses glucose, lactose and raffinose and produces acids, colonies are white, moist and round. Probably *Streptococcus sp*

Twenty four pure cultures (all acid producers) and one mixed culture were introduced from the Soil Microbiology laboratory at the University of Alberta, and their ability to dissolve precipitated phosphate was examined using the previous method. The results are shown in Table 2.

Eleven organisms produced clear zones around their colonies. *Agrobacterium tumefaciens* strain 2, *Pseudomonas sp* (2) and *Enterobacter aerogenes* died three weeks later.

Table: 2 Dissolution of precipitated phosphate in the medium by soil bacteria after 7 days of incubation using (APM)

Code No.	Organism	action*	acid production from glucose
C_1	Agrobacterium radiobacter (1)	-	+
C_2	" " (2)	-	+
C_4	" " (3)	-	+
C_9	" " (4)	-	+
C_{11}	" " (5)	-	+
C_3	" tumefaciens (1)	+	+
C_7	" " (2)	+	+
C_8	" " (3)	-	+
C_{23}	" " (4)	-	+
C_{24}	" " (5)	-	+
C_{10}	" rhizogenes	-	+
C_5	" Sp	+	+
C_6	" "	+	+
C_{15}	Pseudomonas Sp (1)	-	+
C_{16}	" " (2)	+	+
C_{17}	" " (3)	+	+
C_{18}	" syringae	-	+
C_{22}	" stutzeri	+	+
C_{14}	Acinetobacter anitratus	+	+
C_{20}	Enterobacter aerogenes	+	+
C_{21}	Enterobacter Sp	+	+
C_{25}	Mixed Cultures	+	+
C_{12}	Unknown	-	+
C_{13}	" "	-	+
C_{19}		-	+

* - no clearing
\+ clearing zone

Cook F.D. (Special Communication)

Cook F.D. (Special Communication)

Cook F.D. (Special Communication)

The phosphate dissolving bacteria (PDB) isolated from soil and those from the Soil Microbiology laboratory were kept viable by subculturing them every three weeks; they started dying one year later.

The agar plate method (APM) with precipitated phosphate in the medium has the following disadvantages for assessing the ability of organisms to dissolve phosphate minerals:

1. It is not easy to adjust the pH after autoclaving.
2. The insoluble phosphate produced by adding K_2HPO_4 and $CaCl_2$ to the medium after autoclaving is sensitive to small changes in pH.
3. The form(s) of insoluble phosphate produced is (are) not known and are not necessarily good analogues of soil minerals.
4. It is not easy to handle, and there is a high risk of contamination, especially when adjusting the pH.

To overcome the abovementioned difficulties, and to see if the criterion of clearing in precipitated phosphate media selected for organisms capable of dissolving minerals more closely resembling those in the soil, the precipitated phosphate in the medium was replaced by 0.5% hydroxylapatite ($Ca_{10}(OH)_2(PO_4)_6$) or 0.5% dicalcium phosphate ($CaHPO_4$). The plates were then inoculated with PDB which had been previously isolated using precipitated phosphate in the medium, and incubated at 25°C for 14 days. Except for S_{11}, no isolate produced clear zones in media containing either $Ca_{10}(OH)_2(PO_4)_6$ or $CaHPO_4$. Culture S_4 produced clear zones

around its colonies after 7 days of incubation.

These results indicate that the precipitate formed in the medium is chemically and/or physically sufficiently different from $Ca_{10}(OH)_2(PO_4)_6$ or $CaHPO_4$, that the ability to produce clear zones with recently precipitated P in the medium does not mean that the organism has the ability to cause clearing in a medium with hydroxylapatite. Further,the question as to whether the clearing represents phosphate dissolving ability, or a secondary reaction, arises. If it was a secondary reaction, then the clearing represents a relationship between pH and solubility of the compound. This latter is the most probable. Consequently, selection using this criterion may be primarily a general characteristic such as acid production.

According to these results APM with precipitated phosphate in the medium was considered suitable only for rapid isolation and detection of the ability of soil organisms to produce acid and dissolve some insoluble phosphates. This medium was used to reculture the isolates to keep them viable during this study.

Further modifications were introduced to adjust the pH of the medium to minimize the chance of contamination and to ensure uniformity in the media. A potentiometric titration was conducted on the autoclaved media (after the phosphate was precipitated) using sterilized 0.5 N NaOH solution, and a titration curve was constructed (Appendix 1). Using that curve, pH could be precisely adjusted to any desired value

by adding the appropriate volume of sterilized 0.5 N NaOH, providing that the medium was prepared as previously described and the same volume of the medium was used. Several checks were subsequently made to ensure that the desired pH was in fact always achieved.

"Phosphate dissolving bacteria" were kept viable from October 1979 by reculturing them every three weeks on agar with precipitated phosphate. Except for S_4 they all retained their ability to produce clear zones. After several subculturings, the ability of S_4 to produce clearing was restored. Sperber (1957, 1958a) found that some PDB irreversibly lost their ability to dissolve phosphate after subculturing.

4.2 Qualitative Studies on Phosphate Dissolving Bacteria (PDB)

4.2.1 Effect of Medium Contents

To examine the effect of the medium contents on growth and clearing, the folowing media were prepared :

1. Salts (as in section 4.1) and glucose.
2. Salts (as in section 4.1) and glucose plus yeast extract.
3. Salts (as in section 4.1) and glucose plus asparagine.
4. Salts (as in section 4.1) and glucose plus peptone.
5. Salts (as in section 4.1) and glucose plus peptone plus

yeast extract.

6. Salts (as in section 4.1) and glucose plus asparagine plus yeast extract.

Fifteen organisms were tested for their ability to produce clear zones when grown on these media.

The effect of yeast extract on clearing was very definite, with more than half of the organisms failing to produce clear zones when it was omitted (Table 3). The addition of asparagine or peptone had little effect on the ability of these bacteria to dissolve phosphate. These results suggested that most of the organisms tested required substances in yeast extract. This supports the findings of Katznelson and Bose, (1959) that PDB isolated from wheat roots and rhizosphere required substances in yeast extract. In general, there was no difference between the effect of yeast extract alone, yeast extract with asparagine, or yeast extract with peptone, on the solubilization of precipitated phosphate. All cultures, however, with the exception of S_3, S_8 and C_3, produced bigger haloes around their colonies when asparagine or peptone were omitted.

In addition to the effect caused by medium contents, the difference in the size of the clear zone also might be due , in part, to:

1. Bacteria differ in their requirements for,and use of, amino acids. Bacteria which use amino acid as a source of carbon produce ammonia and increase the pH, while those using only glucose might produce acids and lower

Table: 3 Effect of medium contents on clearing by PDB

Culture Code No.	Medium Contents					
	S + G	S + G + Y.E.	S + G + Asp.	S + G + Pept.	S + G + Pept + Y.E.	S + G + Asp + Y.E.
S_1	-	+	-	-	+	+ (L)
S_2	-	+	-	+	+	+ (L)
S_3	-	+ (L)	-	+	+	+
S_4	+ (L)	+	+	-	+	+ (L)
S_5	-	+ (L)	-	-	+ (L)	+
S_6	-	+	-	-	+	+ (L)
S_7	-	+	-	-	+	+
C_3	-	+ (L)	+	+	+	+
C_5	-	+	-	-	+ (L)	+ (L)
C_6	+(L)	+	+	+	+	
C_{14}	+	+	+	+	+	+ (L)
C_{17}	+	+	+	+	+ (L)	+
C_{21}	+	+	+	-	+	+ (L)
C_{22}	-	+	-	+ (L)	+	+
C_{25}	+ (L)	+	+	-	+ (L)	+

S: Salts
G: Glucose
Y.E.: Yeast extract
Asp.: Asparagine
Pept: Peptone
L: Small zone
+: Clearing
-: No clearing

the pH. Bacteria which use both might either decrease or increase the pH depending upon the amount of ammonia and acids produced.

2. The thickness of medium in the plates might vary. The thicker the plates, the more substance (e.g. acids) is required to diffuse to the bottom of the plate to dissolve the phosphate between the top and the bottom of the plate. This would explain some of the variability in the results.

4.2.2 Effect of pH.

The previously described agar plate method with precipitated phosphate in the medium was used to examine the effect of pH on dissolution of insoluble phosphate. Five groups of agar plates with precipitated phosphate were prepared with the following pH values : 5,6,7,8 and 9. The pH was adjusted using the titration curve in Appendix 1. When the pH was adjusted to 4.5 or lower, all the precipitated phosphate dissolved.

Seven plates of each pH group were inoculated with organisms S_1-S_7 separately, and incubated at 25°C. The plates were examined for the presence of clear zones around the colonies after 2,4,5,6,7 and 8 days.

All bacteria grown on the medium with initial pH 5 produced clear zones around their colonies after two days except organism S_3 which produced the clear zone after 6 days (Figure 2). As the initial pH of the media increased,

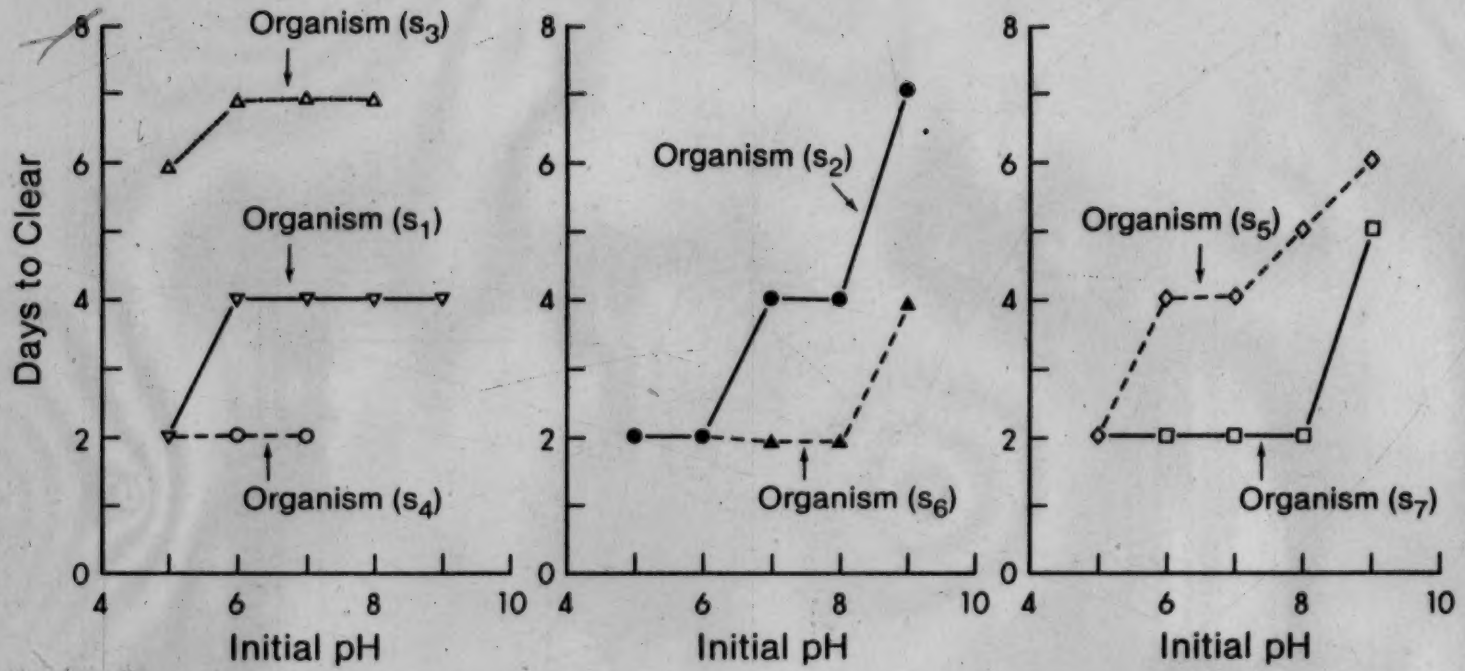

Figure 2. Days required for several PDB to develop clear zones around their colonies at different initial pH values using agar plate method (APM) with precipitated phosphate.

the time needed to detect clearing increased. This is due to two related mechanisms :

1. At higher pH values, the forms of insoluble phosphate formed are more resistant. When the pH increased, first the HPO_4^{--} and finally the PO_4^{---} ions dominated, according to the following equilibrium (Hayman, 1975) :

$$\underset{\text{Acid solutions}}{H_2PO_4^-} \xrightarrow{OH^-} H_2O + HPO_4^{2-} \xrightarrow{OH^-} \underset{\text{Alkaline solutions}}{H_2O + PO_4^{3-}}$$

The distribution of orthophosphate ions in solution as a function of pH is shown in Figure 3.

In the presence of calcium ions, phosphate solubility is controlled according to the following equilibrium:

$$2H_2PO_4^- + 3Ca^{++} \text{------} Ca_3(PO_4)_2 + 4H^+$$

Lindsay (1979) reported that in the pH range of 6 to 6.5 several phosphate minerals, such as brushite ($CaHPO_4.2H_2O$), monomite ($CaHPO_4$), octacalcium phosphate ($Ca_4H(PO_4)_3 2.5\ H_2O$), B - tricalcium phosphate ($Ca_3(PO_4)_2$) and hydroxylapatite ($Ca_{10}(PO_4)_6(OH)_2$) can coexist (Figure 4). As the pH increases the more insoluble forms such as hydroxylapatite dominate. At pH less than 4.5 even hydroxylapatite, the most resistant form, is completely dissolved or transferred to other forms (Lindsay, 1979).

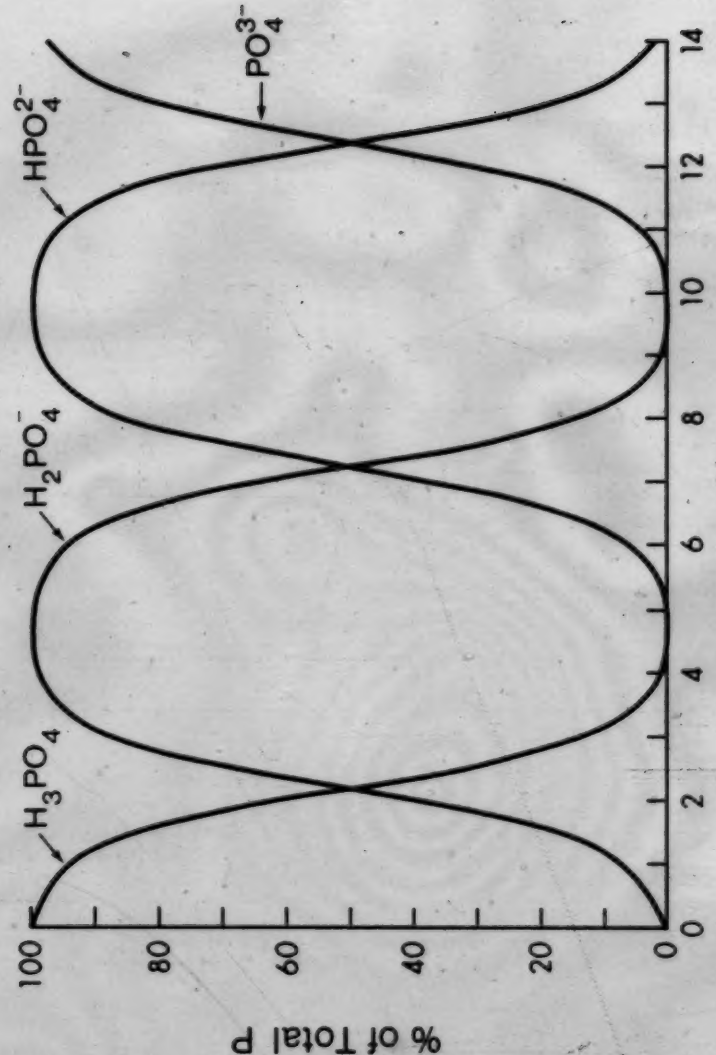

Figure 3. Distribution of orthophosphate species in solution as a function of pH (after Lindsay, 1979).

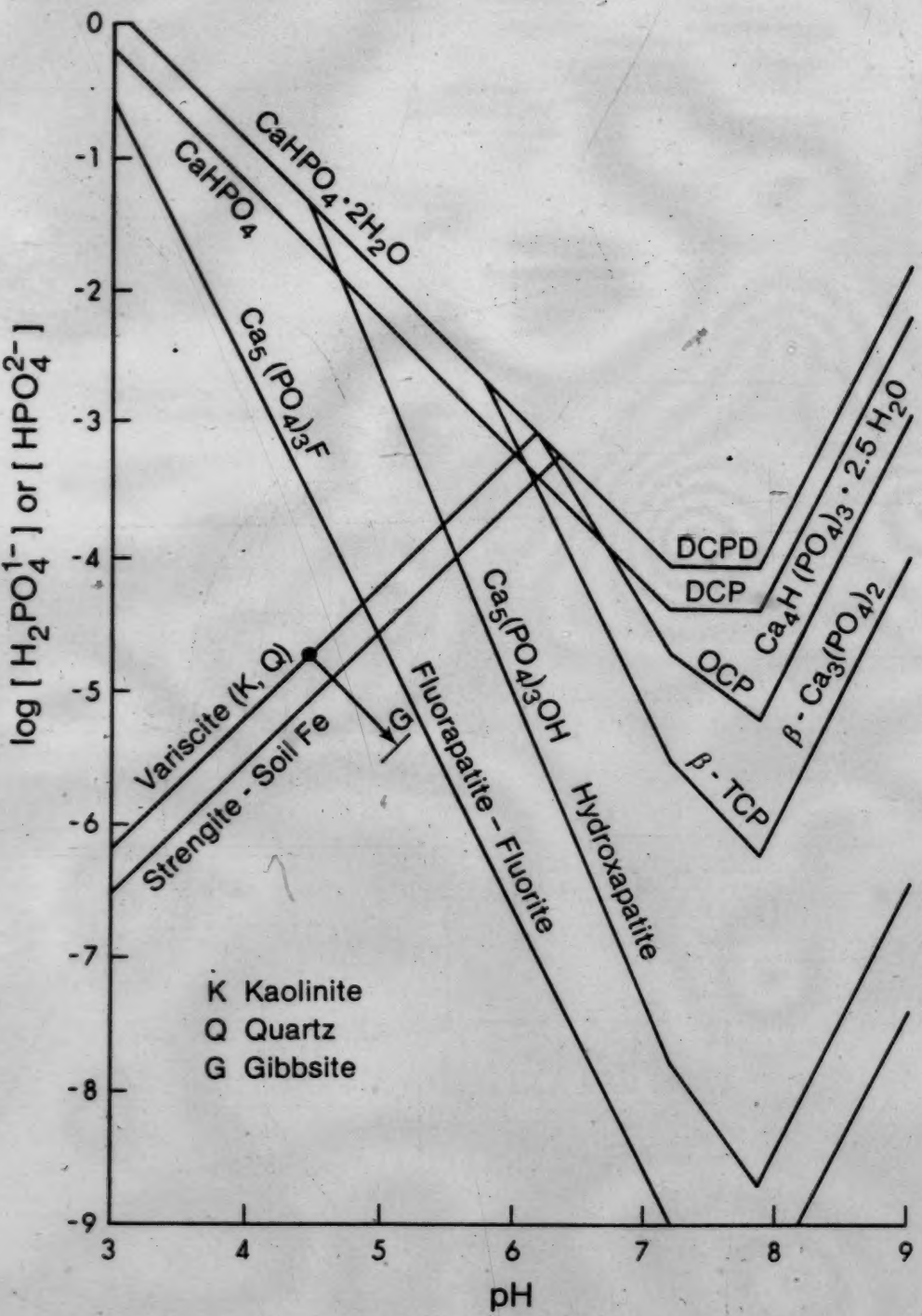

Figure 4. Solubility of different phosphate minerals as a function of pH when Ca^{++} concentration $10^{-2.5}$ or fixed by calcite and CO_2 (g) at 0.0003 atmospheres after Lindsay, 1979).

2. At high initial pH values more acid is needed to lower the pH sufficiently for complete solubilization. Therefore more time is needed to produce the required amount of organic acid.

The time needed to detect solubilization (clearing) varied with the organism used. This may have been a function of both growth rate and metabolic processes affecting the amount of acid produced per unit time.

Organism S. did not produce clearing at either pH 8 or 9 even after 8 days of incubation, whereas S. produced clearing after 8 days when grown on a media of pH 9. These results suggested that these two organisms either produced small amounts of organic acids which were insufficient to cause solubilization, or that at high pH their activity decreased.

From the results of this experiment, it was concluded that an initial pH of 6 to 7, all organisms tested produced clearing of precipitated phosphate media within 7 days.

5. Quantitative Studies on PDB

5.1 Development of Methodology

5.1.1 Preliminary Studies on Media.

A quantitative method was needed to measure the capacity of PDB to dissolve insoluble phosphate minerals. General requirements for this method were :

1. A liquid medium devoid of soluble phosphate and calcium.
2. A known form of insoluble phosphate mineral.
3. Ease of adjustment of the medium pH before autoclaving.
4. The pH of the medium should either not change during autoclaving, or it should change to a known pH value.

To achieve the first requirement, a liquid medium was prepared using the same composition as the agar plate medium which was previously used to isolate PDB, except that the agar and the precipitated phosphate were omitted. Orthophosphate was measured in this medium and found to be 6 ppm - P. When yeast extract was omitted, the concentration of P in the medium was only 0.05 ppm. This indicated that the source of phosphorus was the yeast extract. According to these results, yeast extract should not be added to the medium. In a previous experiment, however, most of the PDB failed to grow and dissolve phosphate minerals when grown on a medium devoid of yeast extract. Therefore, it was considered necessary to retain yeast extract in the medium

although it introduces some soluble phosphorus which was a recognised disadvantage.

To achieve the second condition, three sources of "insoluble phosphate" were examined.

1. A liquid medium (50 ml) + 50 mg of $Ca_{10}(OH)_2(PO_4)_6$ (HA).
2. A liquid medium (50 ml) + 50 mg $CaHPO_4$.
3. A liquid medium (50 ml) + 50 mg of precipitated phosphate (prepared by mixing 10% K_2HPO_4 solution with 10% $CaCl_2$ solution in a 15:25 V:V ratio and then washing the precipitate until it became free of chloride ions).

These media were adjusted to pH 6.6 - 6.8 using 0.5 N NaOH solution. The media were then autoclaved at 120°C and 103 kPa for 40 minutes. Orthophosphate and pH were measured before and after autoclaving.

In all cases the pH was lowered and the concentration of P increased after autoclaving (Table 4). The medium containing HA was the least affected by the autoclaving, and contained the lowest concentration of P after autoclaving. The drop in pH after autoclaving was 0.8, 0.8, 1.8 and 1.4 units in the $Ca_{10}(OH)_2(PO_4)_6$, $CaHPO_4$, precipitated phosphate and control treatments respectively.

From these results it was concluded that $Ca_{10}(OH)_2(PO_4)_6$ was the most appropriate insoluble phosphate source for subsequent experiments because the concentration of P was the least after autoclaving and pH was also the least affected by autocalving.

Table: 4 Effect of autoclaving on pH and concentration of orthophosphate in the media using three different forms of insoluble phosphate

Form of insoluble P	pH of the media		Concentration of P (ppm)	
	B. Aut.	A. Aut.	B. Aut.	A. Aut.
$Ca_{10}(OH)_2(PO)_6$ (Hydroxylapatite)	6.6 ± 0.00	5.8 ± 0.03	4.60 ± 0.23	7.20 ± 0.5
$Ca\ HPO_4$ (Dicalcium phosphate)	6.7 ± 0.10	5.9 ± 0.08	19.5 ± 1.5	34.0 ± 1.8
Precipitated P	6.8 ± 0.03	5.0 ± 0.01	260 ± 6.4	340 ± 1.7
Control	6.6 ± 0.00	5.2 ± 0.04	4.3 ± 0.10	4.40 ± 0.15

B. Aut. = Before autoclaving
A. Aut. = After autoclaving

A way to adjust the pH to avoid contamination and to arrive at pH 7.0 after autoclaving was required. A liquid medium was prepared as previously described, omitting the P source, and 50 ml were dispensed into each of 15 erlenmeyer flasks (125 ml) containing 50 mg of $Ca_{10}(OH)_2(PO_4)_6$. The 15 flasks were divided into 5 groups of 3 replicates each. The pH of each group was adjusted before autoclaving to a known value, and was measured again after autoclaving.

Autoclaving caused a constant drop of one pH unit in each case (Table 5). Based on these results, the pH of the medium was subsequently adjusted to one pH unit higher than desired prior to autoclaving. Several checks were made throughout the studies that follow to ensure that the desired pH was indeed obtained after autoclaving.

5.1.2 Fate of Ca and P from $Ca_{10}(OH)_2(PO_4)_6$ during incubation with PDB.

The previously described liquid medium was prepared and 44 ml were dispensed into separate 125 ml erlenmeyer flasks, each containing 50 mg of $Ca_{10}(OH)_2(PO_4)_6(HA)$. The medium in each flask was adjusted to pH 8.0 using 0.5 N NaOH solution. The flasks were then autoclaved at 120°C and 103kPa for 40 minutes. After autoclaving, pH was checked in seven flasks and found to range from pH 7 - 7.05 indicating that the desired pH was consistently attained. Using a sterilized loop, inocula were transferred, from three day old cultures grown on agar plates, to tubes containing 6 ml of autocalved

Table: 5 Effect of autoclaving on change in pH over a range of initial pH values in media containing 50 mg $Ca_{10}(OH)_2(PO_4)_6$ as a source of insoluble phosphate

pH before autoclaving	pH after autoclaving	change in pH due to autoclaving
8.4	7.3 ± 0.09	-1.1
8.3	7.2 ± 0.05	-1.1
8.0	7.0 ± 0.00	-1.0
8.0	7.0 ± 0.07	-1.0
8.2	7.2 ± 0.01	-1.0

liquid medium containing no HA. The turbidity of the inoculum was measured using a Klett - Summerson Photoelectric Colorimeter Scale with a green filter and adjusted to read between 55 - 70 units. The contents of each tube were then transferred to the corresponding flask. The flasks were incubated at 25°C on a rotary shaker at 250 rpm for 10 days. At the end of the incubation period, pH of the flasks was measured. The contents of each flask were then centrifuged at R.C.F. 17,300 and washed three times with 0.85 % NaCl solution, the supernatants collected in 100 ml volumetric flasks, and orthophosphate and calcium measured.

All organisms increased the amount of P in solution over control. Some organisms raised the pH after 10 days of incubation, while others lowered it (Table 6). Those organisms which lowered the pH, brought more phosphorus and calcium into solution from the insoluble $Ca_{10}(OH)_2(PO_4)_6$ than those which increased the pH. The Ca^{++}/P ratio in solution was greater than the measured Ca^{++}/P ratio in $Ca_{10}(OH)_2(PO_4)_6$ for all the organisms tested except C_{23}. The increase in Ca^{++}/P ratio was more pronounced in those organisms which raised the pH (Table 6). Organisms S_4, C_7, and C_{21} raised the pH to 8.3, 8.5 and 7.6 respectively. The Ca^{++} (in solution)/P (in solution) ratio was 5.5, 7.8 and 6.9 for these organisms respectively. The Ca^{++}/P ratio (in solution) for the organisms which lowered the pH was between 2.4 - 3.7. For organism C_{23} the ratio was 2.03 which is lower than 2.15; the Ca^{++}/P ratio in $Ca_{10}(OH)_2(PO_4)_6$. C_{23},

Table 6: Ca^{++} and orthophosphate in solution following 10 days incubation of some phosphate dissolving bacteria with 50 mg $Ca_{10}(OH)_2(PO_4)_6$/50 ml medium

Organism	pH	mg P / flask	mg Ca^{++} / flask	Ca^{++}/P ratio	mg P calculated	$\frac{\text{mg P measured}}{\text{mg P calculated}}$
S_1	4.4	3.23 ∓ 0.23	7.39 ∓ 0.23	2.37 ∓ 0.03	3.45 ∓ 0.15	0.94
S_3	4.2	3.98 ∓ 0.18	9.57 ∓ 0.30	2.41 ∓ 0.04	4.45 ∓ 0.14	0.89
S_4	8.3	0.47 ∓ 0.04	2.59 ∓ 0.25	5.50 ∓ 0.30	1.19 ∓ 0.07	0.40
S_5	4.7	2.14 ∓ 0.14	6.14 ∓ 0.61	3.04 ∓ 0.02	2.87 ∓ 0.29	0.76
C_3	6.6	1.46 ∓ 0.18	5.26 ∓ 0.30	3.66 ∓ 0.07	2.44 ∓ 0.14	0.60
C_5	4.9	1.22 ∓ 0.10	3.51 ∓ 0.19	2.89 ∓ 0.18	1.64 ∓ 0.09	0.74
C_{14}	4.3	4.49 ∓ 0.13	11.3 ∓ 0.21	2.52 ∓ 0.02	5.25 ∓ 0.61	0.86
C_{17}	8.5	0.33 ∓ 0.01	2.60 ∓ 0.03	7.83 ∓ 0.50	0.71 ∓ 0.02	0.47
C_{21}	7.6	0.26 ∓ 0.01	1.78 ∓ 0.03	6.93 ∓ 0.26	0.83 ∓ 0.01	0.31
C_{22}	4.5	3.95 ∓ 0.18	8.02 ∓ 0.50	2.03 ∓ 0.30	3.73 ∓ 0.23	1.06
Control	6.7	0.33 ∓ 0.004	0.62 ∓ 0.06			

Ca^{2+}/P ratio in $Ca_{10}(OH)_2(PO4)6$=2.15

mg P Calculated = mg Ca^{++} measured/2.15 (assuming that all the solubilized P stayed in solution)

however, showed very little growth. These results suggested that those cultures which raised the pH may have used the released phosphorus to produce new cells while those which lowered the pH produced more waste and probably more organic acids, which lowered the pH, causing more solubilization.

It was concluded from these results, that the amount of P dissolved was underestimated when only orthophosphate in solution was considered; and that further, soluble orthophosphate is not a valid sole parameter of measuring the role of bacteria in cycling of P from sparingly soluble mineral forms. No attempts to draw up a P balance sheet during dissolution experiments have been reported in the literature. The data in the literature use only the amount of P in solution as an indicator of the ability of various organisms to dissolve sparingly soluble phosphate minerals.

The fate of P added as $Ca_{10}(OH)_2(PO_4)_6$, at the end of the incubation period can be illustrated as follows :

$$Pt = Psi + Pso + Pc + Pr$$

where

Pt = Total amount of P added

Psi = inorganic P in solution

Pso = organic P in solution

Pc = P in cells (biomass - P)

Pr = undissolved P

In order to assess the capacity of these organisms to dissolve $Ca_{10}(OH)_2(PO_4)_6$ and to follow the fate of dissolved P, biomass - P as well as soluble P should be determined.

From an ecological and agricultural perspective, the importance of organic P in the cells must be considered because soil solutions seldom contain more than 1 ppm - P.

5.1.3 Measurement of P in Biomass

To measure biomass - P, the cells must be separated from the undissolved $Ca_{10}(OH)_2(PO_4)_6$. Three methods, to accomplish this separation, were examined.

1. Sodium Bicarbonate Method.

In this method the cells and the undissolved $Ca_{10}(OH)_2(PO_4)_6$ at the bottom of the tube after the separation of the solution (Psi + Pso) (Figure 5), were treated with 5ml of $NaHCO_3$ solution at pH 8.5. $NaHCO_3$ was used in an attempt to help disperse bacterial cells which are negatively charged (Marshall, 1980). The contents of the tube were then suspended using a vortex mixer. The suspension of the cells and the residual $Ca_{10}(OH)_2(PO_4)_6$ particles were centrifuged at R.C.F. = 121 for 10 minutes to settle the apatite particles. The suspended materials, considered to be cells, were then transferred into a 50 ml erlenmeyer flask. The above treatment was repeated several times until the supernatant was clear. This was considered an indication that all the cells were separated from the undissolved $Ca_{10}(OH)_2(PO_4)_6$. The residue was then dissolved in HCl and phosphorus determined. The cells were digested and phosphorus determined in the digest.

2.Chloroform Method.

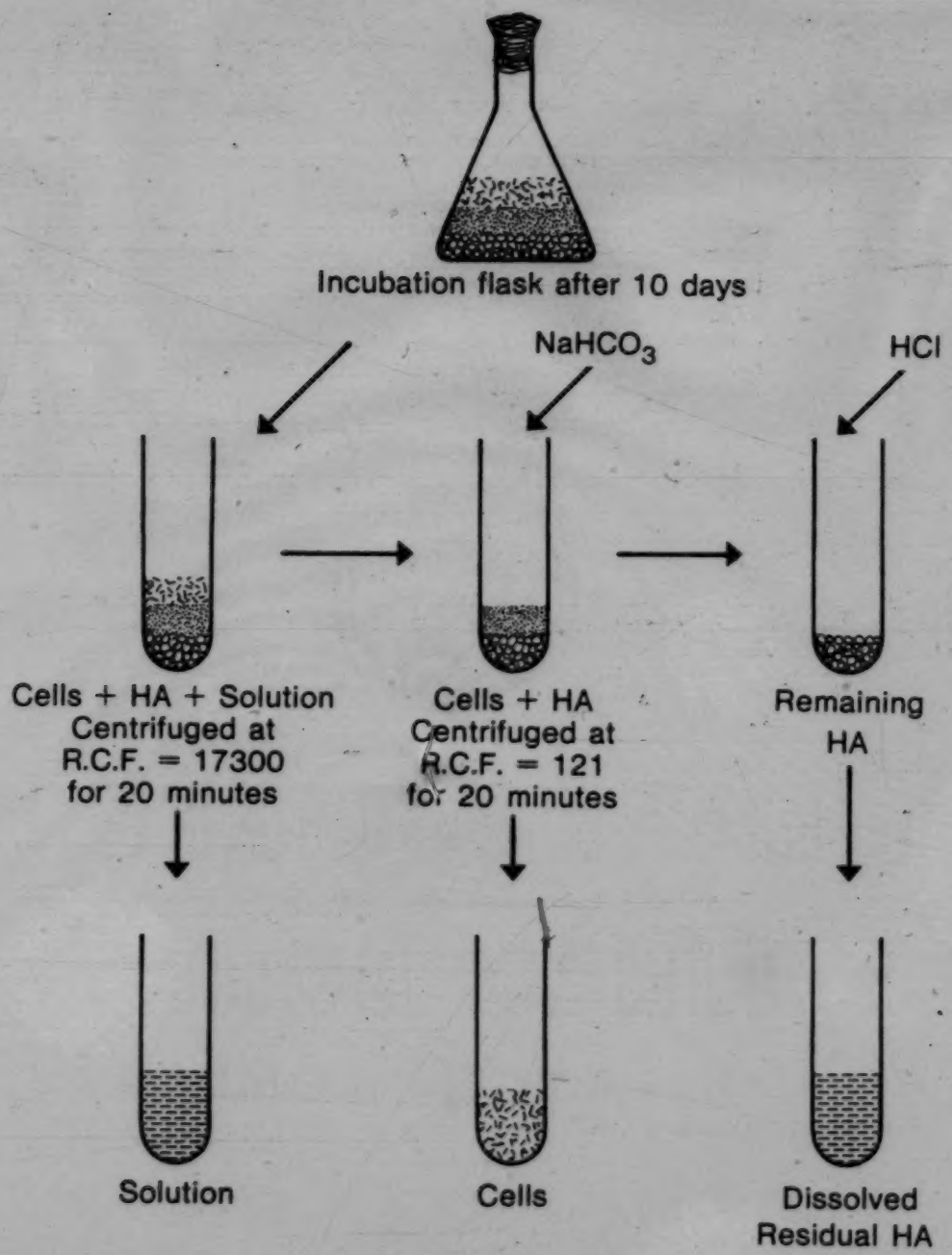

Fig. 5. Separation ofcells from undissolved $Ca_{10}(OH)_2(PO_4)_6$ particles using $NaHCO_3$ Method.

In this method the contents of each flask after separation of the solution were treated in the same way as in the sodium bicarbonate method, except using 5 ml of chloroform instead of sodium bicarbonate. The role of chloroform was to lyse the cell and release microbial P (Hedley and Stewart, 1980a).

3.HCL Method.

In this method the cells and the undissolved $Ca_{10}(OH)_2(PO_4)_6$ particles were treated with 5 ml of 0.5 N HCl to dissolve the residual $Ca_{10}(OH)_2(PO_4)_6$ (Figure 6). The contents were then centrifuged at R.C.F.= 17,300 for 20 minutes and the supernatant transferred into a 100 ml volumetric flask. The cells, remaining at the bottom of the centrifuge tube, were washed three times with 15 ml of 0.85 % NaCl solution. Phosphorous content of the HCl solution and of the cells was then determined. The medium used to examine these methods was that developed previously. The organisms examined were S_4, C_{17} and C_{23}. After 10 days of incubation the solution was separated by centrifuging the contents at R.C.F. = 17,300.

Separation of the cells and the undissolved $Ca_{10}(OH)_2(PO_4)_6$ particles using the chloroform method was unsuccessful because the cells and the undissolved particles could not be separated after the addition of chloroform. With the HCl and $NaHCO_3$ methods, separation of cells from undissolved particles appeared to be successful.

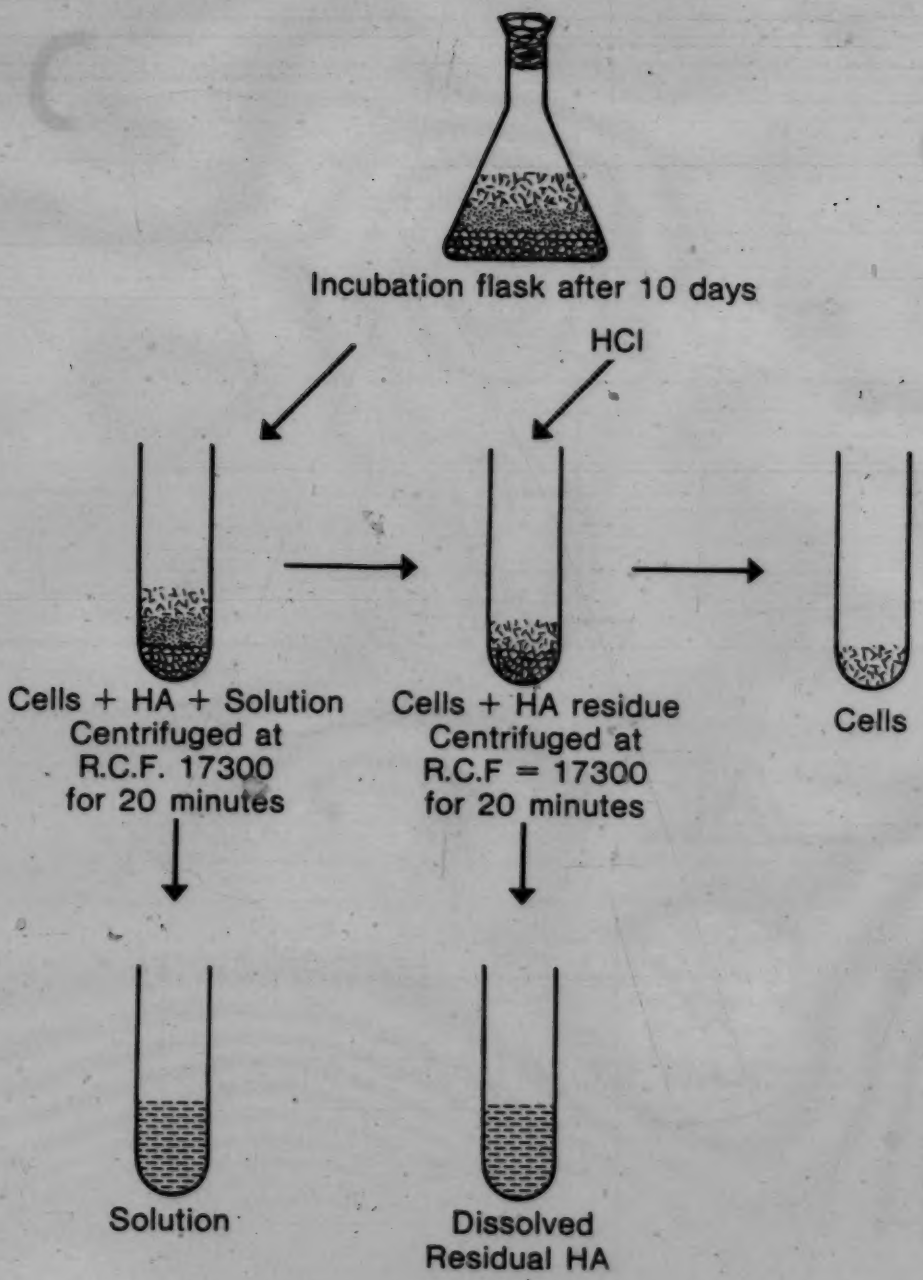

Fig. 6. Separation of cells from undissolved $Ca_{10}(OH)_2(PO_4)_6$ particles using HCl method.

In general, the amount of P recovered was higher when HCl was used than when $NaHCO_3$ was used (Table 7). The amount of biomass - P measured in S_4, C_1, and C_2 was 0.73, 0.41 and 0.036mg respectively, when the HCl method was used. It was 2.59, 3.87 and 0.052mg respectively when the $NaHCO_3$ method was used. These data suggest that either HCl released most of the biomass - P during $Ca_{10}(OH)_2(PO_4)_6$ dissolution, or $NaHCO_3$ included some $Ca_{10}(OH)_2(PO_4)_6$ particles with the cells, either in solution or as occluded particles. The 0.5 N HCl solution, however, is unlikely to release much of the biomass - P. The $NaHCO_3$ in the digestion flask further makes it difficult to add the digestion solution, because of the violent reaction between $NaHCO_3$ and the acid.

It was concluded that the HCl method is a simple and more reliable method for separating cells from hydroxylapatite particles. Further examination of the HCl method as it affected cells was warranted, prior to using the method on a routine basis.

5.2 Disposition of P in cultures of phosphate dissolving bacteria.

5.2.1 Effect of HCl on P content of cells.

Table: 7 Fate of P from insoluble phosphate after 10 days of incubation of three PNB with $Ca_{10}(OH)_2(PO_4)_6$ as a sole source of insoluble P. Original volume of the medium was 50 ml.

Organism	pH	mg P/flask					% P recovered
		Psi	Pr	Pc	Pt	P	
1 - HCl Method							
S_4	8.2	0.460 ± 0.01	7.40 ± 0.17	0.730 ± 0.020	8.59	10.0	86
C_{17}	8.5	0.420 ± 0.06	8.10 ± 0.07	0.410 ± 0.008	8.93	9.93	90
C_{22}	4.4	4.88 ± 0.29	4.20 ± 0.10	0.036 ± 0.001	9.12	9.98	91
Control	6.6	0.160 ± 0.05	9.02 ± 0.25	Nil	9.18	10.1	91
2 - $NaHCO_3$ Method							
S_4	8.2	0.460 ± 0.01	5.20 ± 0.58	2.59 ± 0.180	8.24	9.86	84
C_{17}	8.5	0.420 ± 0.06	4.12 ± 0.39	3.87 ± 0.420	8.40	9.93	85
C_{22}	4.4	4.88 ± 0.29	2.94 ± 0.26	0.052 ± 0.003	7.87	9.95	79
Control	6.6	0.160 ± 0.05	6.68 ± 0.14	0.101 ± 0.290	7.08	10.0	69

Psi = inorganic P in solution
Pt = Total P recovered
Pr = undissovled P
P = P added as $Ca_{10}(OH)_2(PO_4)_6$
Pc = biomass-P

5.2.1.1 Materials and Methods.

Two experiments were conducted to test the hypotheses that :

1. P release from cells by HCl is a function of the P content of the medium or the cells.
2. P release from cells by HCl is a function of age of the culture.

Organisms S_4, C_{14} and C_{21} were used. In the previous experiments, S_4 and C_{21} raised the pH of the culture and C_{14} lowered the pH of the culture after 10 days of incubation. The effect of HCl on P content of cells grown in media containing various concentrations of P was examined to determine if the effect of HCl on cellular P was a function of the P concentration in the cells.

Liquid media,as previously described, were used with 5 different P concentrations (5, 25, 65, 105 and 505 ppm of P) and were designated medium number 1,2,3,4 and 5 respectively. P was added as K_2HPO_4 except in medium 1, where the source of P was yeast extract. Media no.2, no . 3, no.4 and no.5 were used with organism S_4, which was examined first, and on the basis of the results, it was decided to use media no. 1, no.2, no.3 and no. 4 with organisms C_{14} and C_{21} . The pH before autoclaving was adjusted to 8.4,8.00,7.6,7.4 and 7.4 in the five media respectively. The pH of the media was adjusted to these different values, because it was found that the more K_2HPO_4 added, the less the change in pH due to autoclaving. Prior to autoclaving,

44ml of the various media were dispensed into separate 125ml erlenmeyer flasks (15 flasks for each treatment). The flasks were autoclaved at 120°C and 103 kPa for 40 minutes. After autoclaving, the pH values were checked in 3 flasks of each medium and were found to be 7.3, 7.4, 7.4, 7.4 and 7.5 in media 1 to 5 respectively. The flasks were then inoculated and incubated as described above. Each treatment was replicated four times. After 10 days of incubation the flasks were removed, the pH was measured and the solution separated by centrifugation. Five ml of 0.5 N HCl were added to the cells in each centrifuge tube, the contents mixed with a vortex mixer, and again centrifuged at R.C.F. = 17,300 for 20 minutes. The supernatant (P released by HCl = PH) was collected in 50 ml volumetric flasks, the cells were then washed three times with 0.85 % NaCl solution, dried at 70°C for 24 hours and weighed. Psi, Pc (biomass-P - PH), PH and Pso were measured as previously described.

As the highest uptake of P by the three organisms occurred when 105 ppm P was added to the medium, it was decided to use this level of P in the next experiment to measure the effect of culture age on amount of P released from cells by HCl.

In this experiment, 0.56 g of K_2HPO_4 was added to 1 litre of the previously described liquid medium (medium no.1 in the previous experiment) to bring the concentration of P to 105 ppm. The experiment was carried out as previously described, except ppm. The experiment was carried out as

previously described, except flasks were removed from the shaker after six different periods of incubation.

5.2.1.2 Results and Discussion

The amount of P released by HCl was independant of P content of the cells (Figures 7,8,9). In general, the amount of P released by HCl from the cells of S_4 was almost the same as from C_{21} when grown in media with more than 25 ppm of P initially present (Figures 7 and 8). The amount of P released from C_{14} cells was independant of P concentration in the medium, but was about one third the amount released from either S_4 or C_{21} when grown on media containing the same concentration of P (Figure 9).

In a separate experiment C_{21} maintained a high population ($6*10^7$ cell ml^{-1}) during 10 days incubation, while C_{14} died after only 4 days (Figure 10), presumably because of the acidity of the media as a result of organic acid production. When 75 mg of $CaCO_3$ were added to the medium, to buffer the system, C_{14} lowered the pH to 7.6, 7.4, 6.6, 6.9, 6.7 and 6.0 after 1, 2, 3, 5, 7 and 12 days of incubation respectively. Under these conditions, C_{14} maintained a high number of cells ($6.3*10^7$ cells ml^{-1} after 12 days. Culture C_{21} changed the pH of the medium to 6.4, 5.6, 6.8, 7.8, 7.7 and 7.9 after the same incubation period, and maintained $2.4*10^7$ cell ml^{-1} after 12 days. [1] The results of this experiment showed that cultures C_{21} and S_4

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[1] Daniel Heaney, Department of Soil Science, University of Alberta.

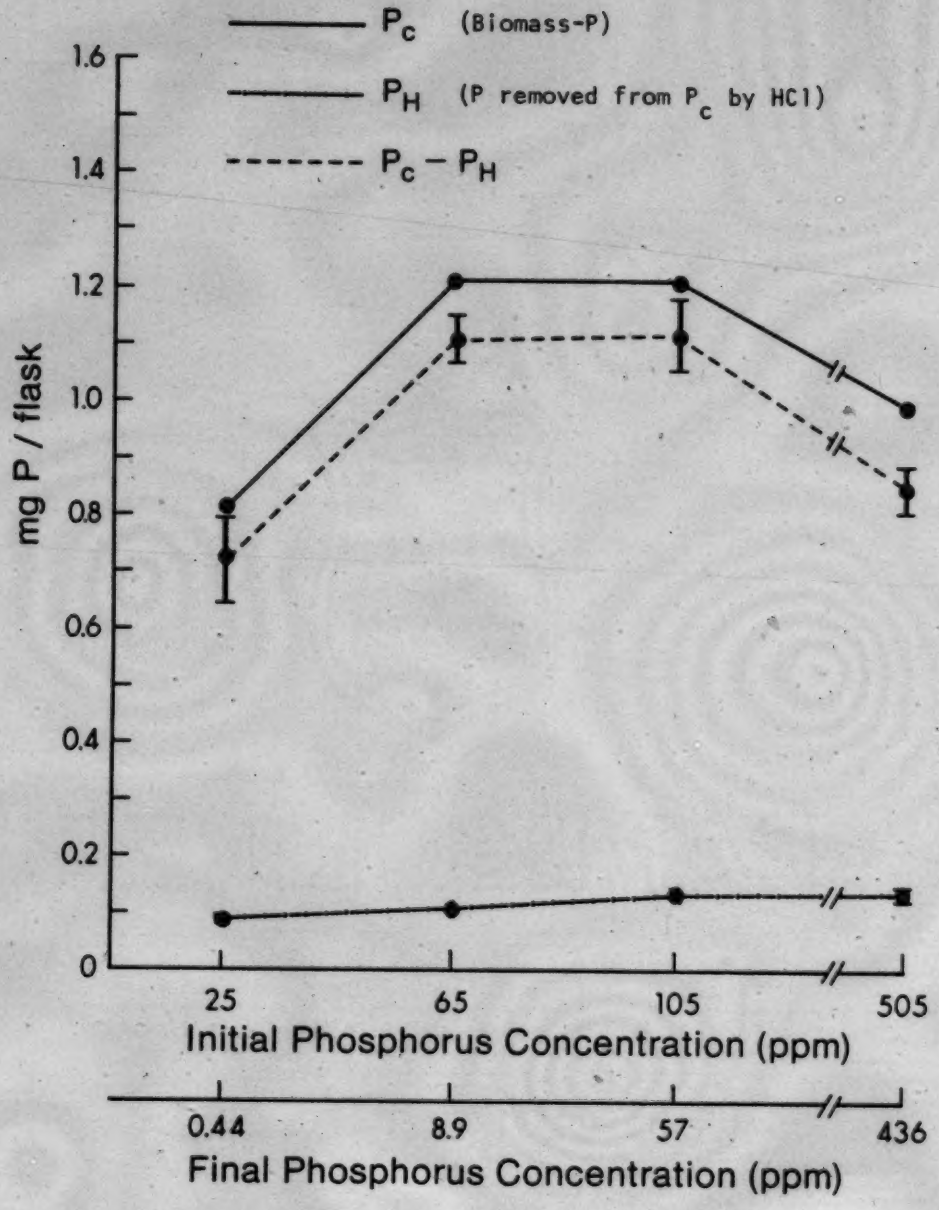

Figure 7. Effect of HCl on P content of the cells of culture S_4 after 10 days of incubation with different initial concentrations of P in the medium.

Unless otherwise stated, standard deviations which are not shown on the graphs have been omitted because they are too small.

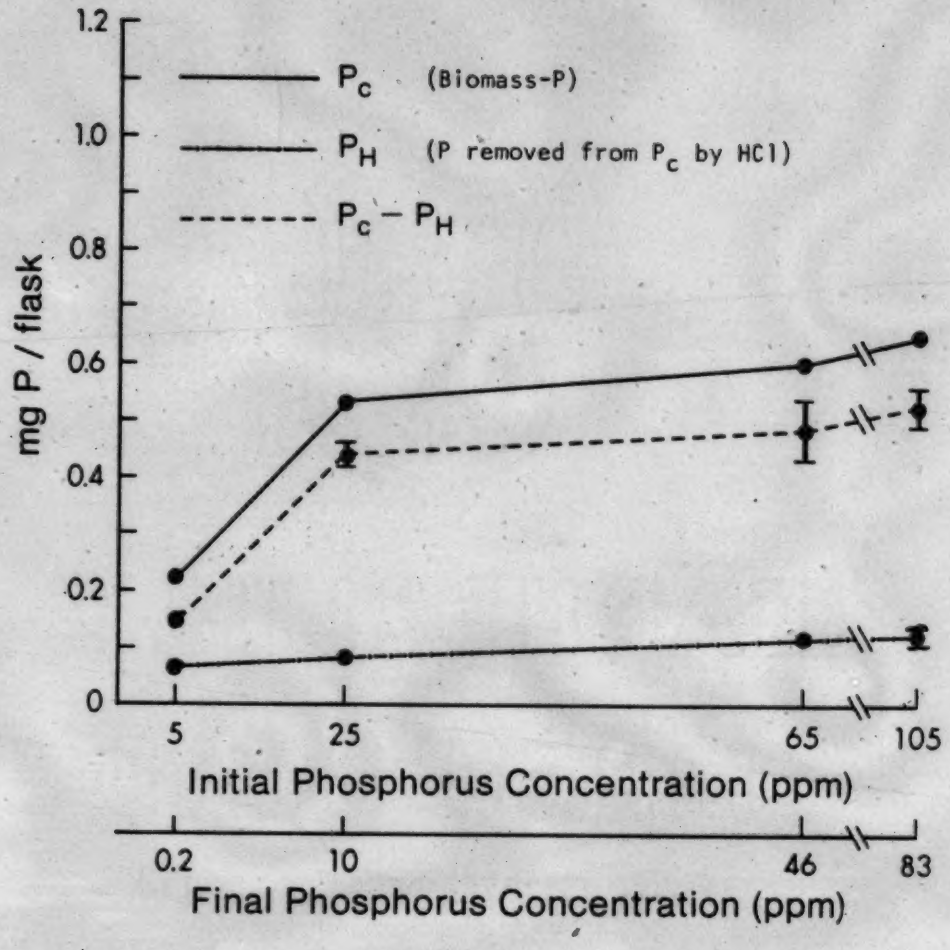

Figure 8. Effect of HCl on P content of the cells of culture C21 after 10 days of incubation with different initial concentrations of P in the medium.

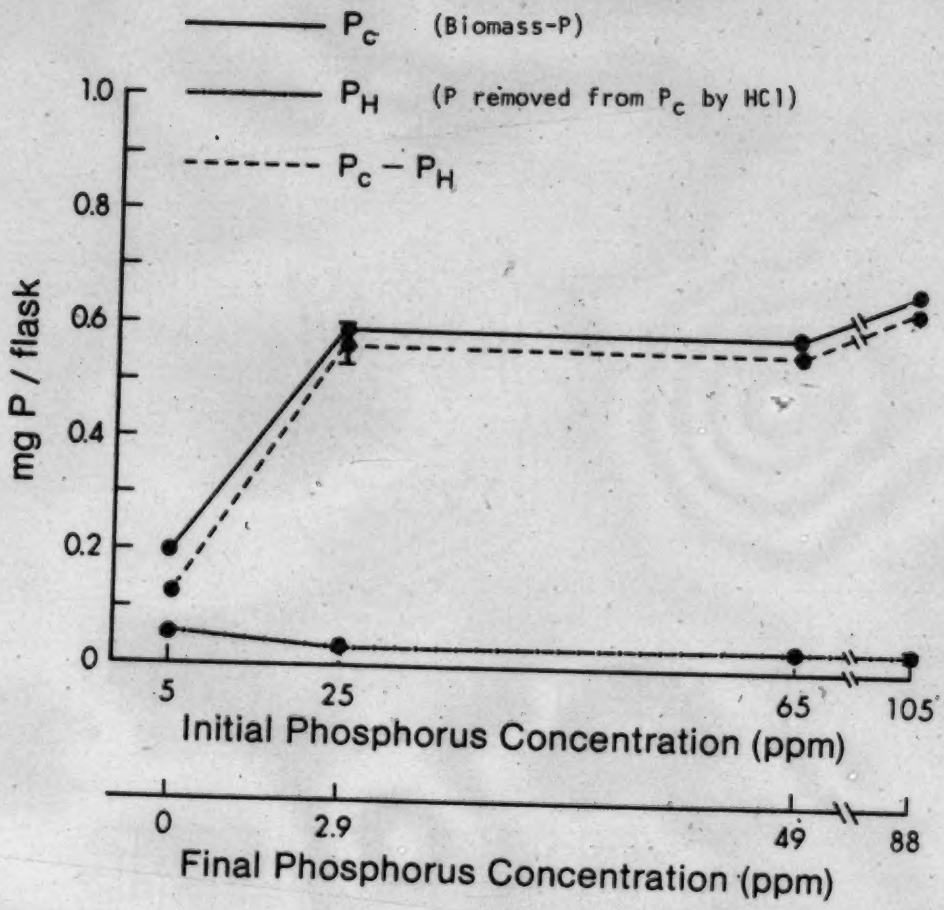

Figure 9. Effect of HCl on P content of the cells of culture C_{14} after 10 days of incubation with different initial concentrations of P in the medium.

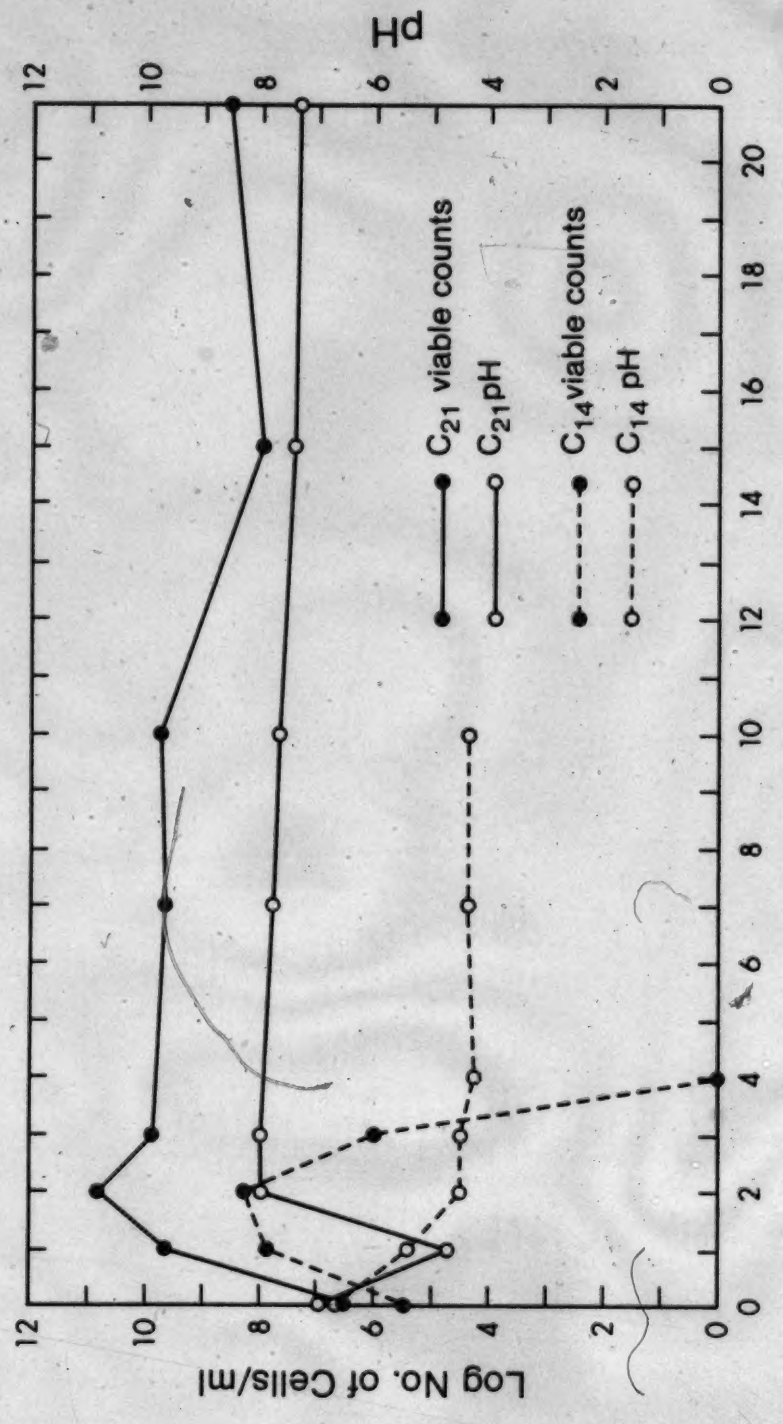

Figure 10. Viable counts and pH. The source of P was HA.

initially lowered the pH of the medium to 3.9 and 4.5 after 1 and 4 days respectively, only to be followed by an increase. The pH of culture $C_{1,4}$, however, was maintained at 4.2 during the experiment (Figure 11).

The low values of Ph (4 % of Pc) in $C_{1,4}$ compared to the higher values (8% - 32% of Pc) in $C_{2,1}$ and S_4 (Table 8) were probably due to the death of $C_{1,4}$ cells after four days of incubation and the release of some of the stored phosphate from the cell into the Pso pools. Luxury consumption and storage of P as polyphosphate is common in bacteria (Harold,1966; Elliott *et al*, in press).

The amount of P released from S_4 cells was reasonably independant of time of incubation (Figure 12). On days 15 and 21, the amount of P decreased by about one third. In the first day, however, the amount of P released by HCl was insignificant (0.017 mg P) compared to the amount of P released in the following days. This was because biomass - P in the first day was much less (0.149 mg P) than biomass - P in the following days (2.27 - 0.971 mg). The released P from S_4 cells, as a proportion of biomass - P, varied between 11 and 25 % (Table 9).

In general, the results for $C_{1,4}$ showed a dramatic decrease in the amount of P released by HCl after 4 days incubation (Figure 13). This, presumably, was because of the death of the cells and the release of P as mentioned earlier. The amount of P released was between 19.5 and 3.3 % of the biomass - P (Table 9). The amount of P released by

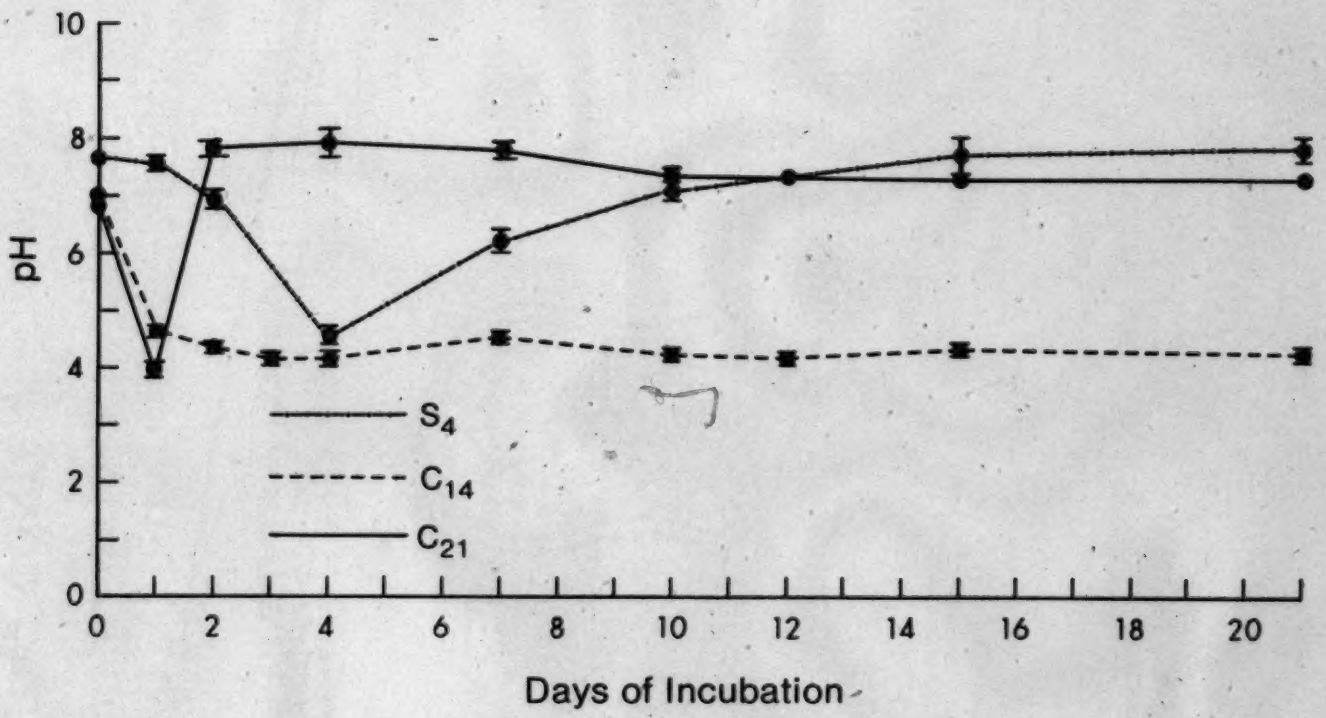

Figure 11. pH of the medium after different incubation periods with cultures C_{21}, C_{14} and S_4. Composition of the medium: glucose, asparagine, yeast extract, salts and 105 ppm P as K_2HPO_4. Standard deviations on days 12, 15, and 21 on C_{21} line are zero.

Table: 8 % P released by HCl from the cells of cultures C_{14}, C_{21} and S_4 after 10 days of incubation with different P concentrations in the media.

Initial P (ppm)	% P released		
	Culture S_4	Culture C_{14}	Culture C_{21}
05	-	27	32
25	11	4	16
65	8	4	19
105	11	4	19
505	14	-	-

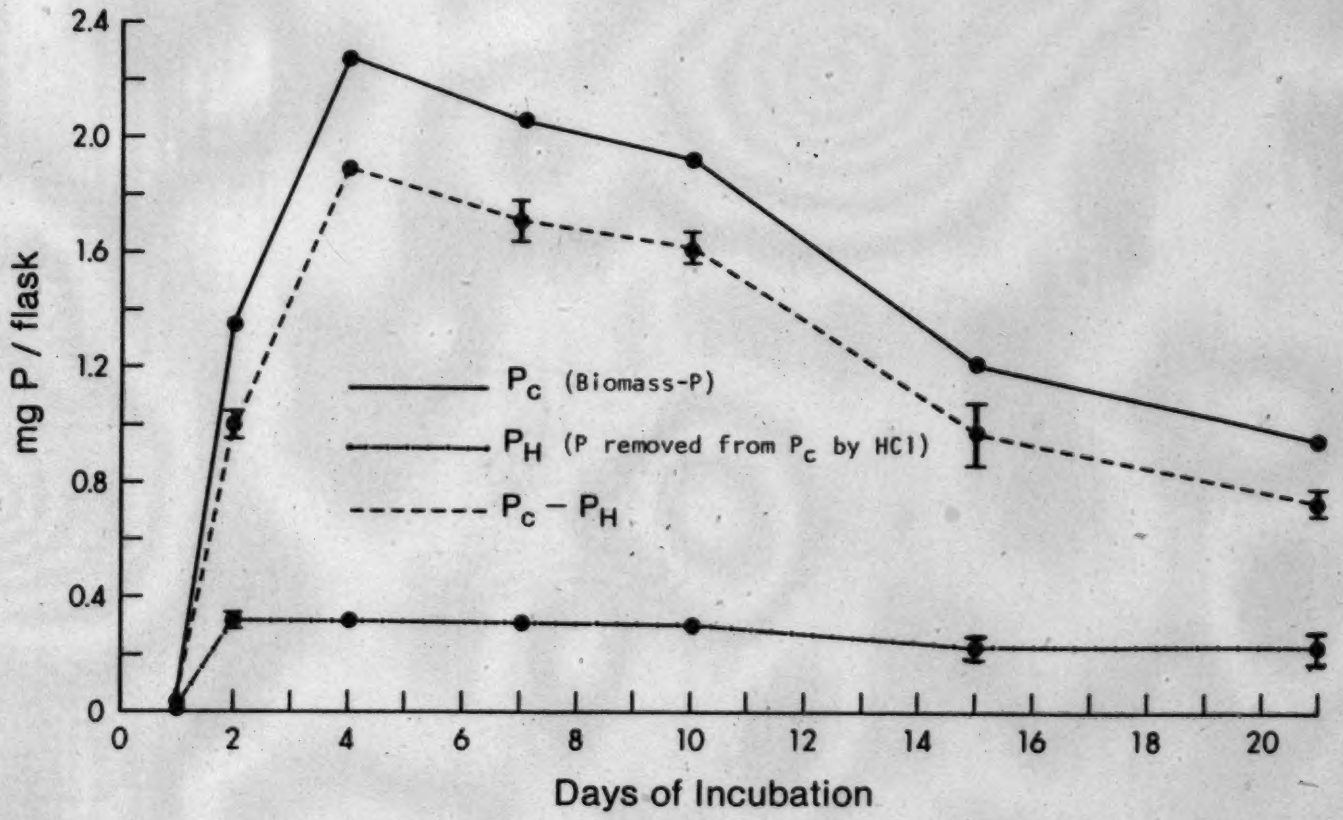

Figure 12. Effect of HCl on P content of the cells of culture S_4 after different periods of incubation. Original concentration of P in the medium was 105 ppm. Only one sample was analyzed for P_H and P_C after 4 days.

Table: 9 % P released by HCl from the cells of cultures C_{14}, C_{21} and S_4 at different times of incubation. Concentration of P in the original medium was 105 ppm

Days of incubation	% P released		
	Culture S_4	Culture C_{14}	Culture C_{21}
1	11	20	17
2	24	17	17
3	-	16	17
4	15	8	17
7	16	10	16
10	16	3	17
12	-	4	16
15	19	3	16
21	25	4	8

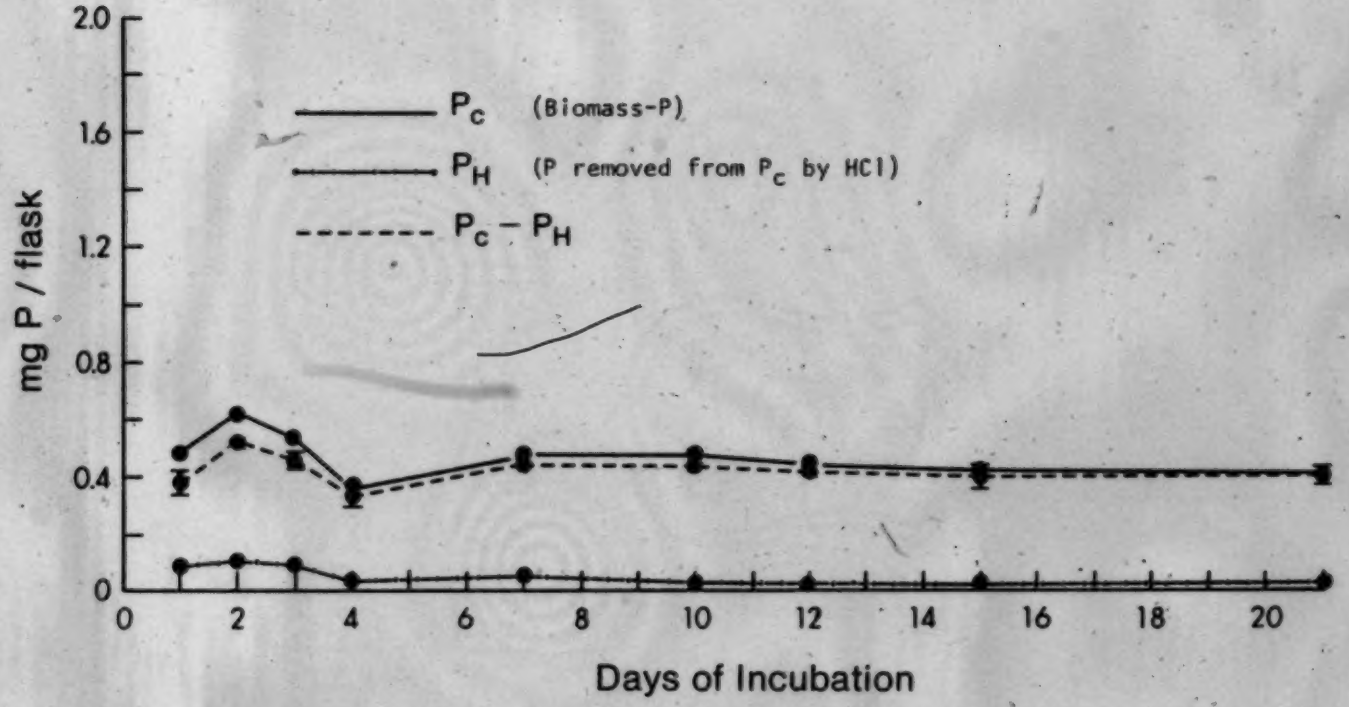

Figure 13. Effect of HCl on P content of the cells of culture C_{14} after different periods of incubation. Original concentration of P in the medium was 105 ppm.

HCl from C_{21} cells gradually decreased from 0.172 mg P on the second day to 0.019 mg P on day 21 (Figure 14). This decrease corresponded to a decrease in biomass - P as well as in dry weight of the cells. The amount of P released as a percentage of biomass-P, however, was almost constant during the incubation period (Table 9).

In general, changes in the amount of P released by HCl corresponded to changes in biomass - P in all three organisms. The amount of P released from S_4 was much greater than the amount released from either C_{14} or C_{21}, because biomass - P of S_4 was greater than that of either C_{14} or C_{21}.

Previous data showed that the use of the HCl method to separate biomass gave lower biomass - P values than did the $NaHCO_3$ method. The hypothesis that HCl released a portion of the cell - P was supported by the data from these two experiments. Release of P from the biomass by HCl appeared to vary between 3 and 25 % of the biomass - P depending on the organism, to be independent of P supply in the medium (above 25 ppm P), P content of the cell, and age of the culture if it was sampled after 4 days. Therefore, the HCl method was considered to be acceptable for use on a routine basis in subsequent work to separate biomass and undissolved $Ca_{10}(OH)_2(PO_4)_6$ particles. The underestimation of biomass - P was recognized but considered to be within acceptable limits.

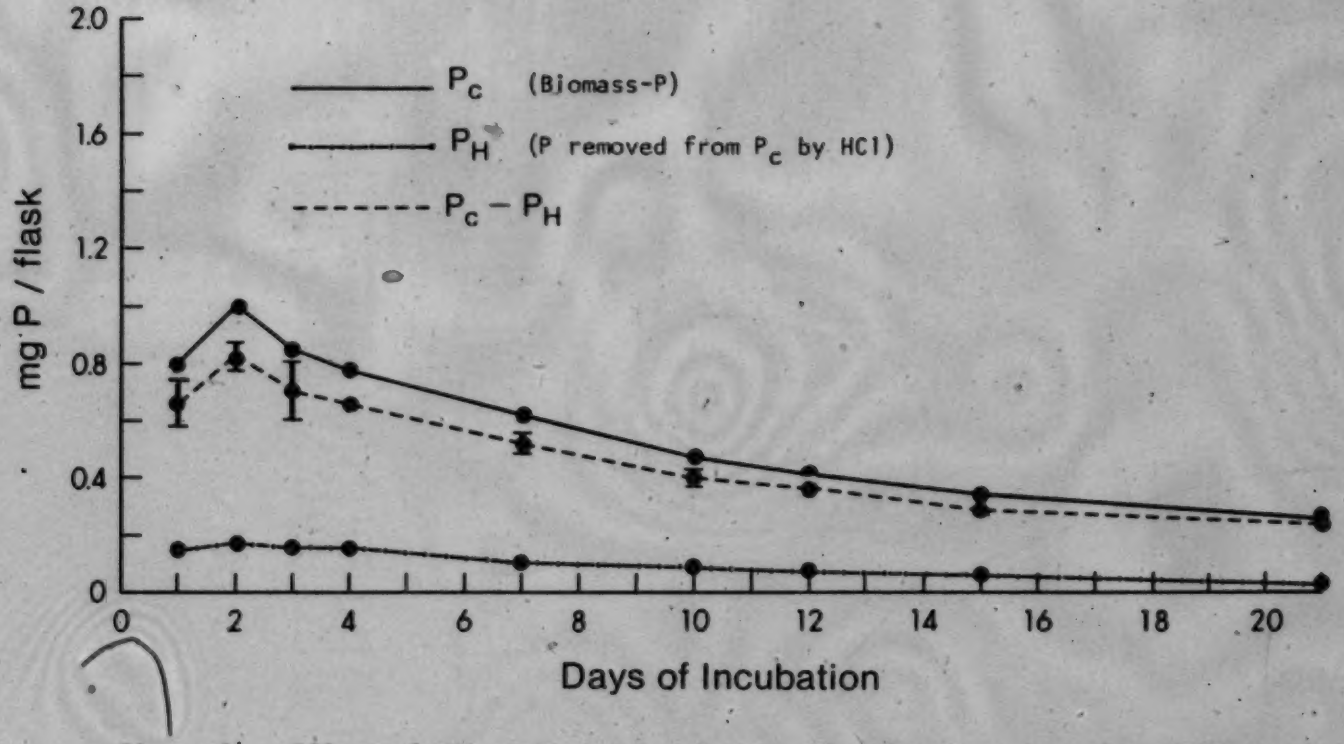

Figure 14. Effect of HCl on P content of the cells of culture C_{21} after different periods of incubation. Original concentration of P in the medium was 105 ppm.

5.3 Uptake of Phosphorus by Cultures of Phosphate Dissolving Bacteria.

Phosphorus uptake [1] and percentage of P in bacterial cells (dry basis) were calculated from the previous data.

For any given treatment, biomass - P of $C_{1,4}$ was almost the same as that of $C_{2,1}$ (Table 10). When grown in a medium rich in phosphorus (more than 25 ppm - P), biomass - P of both these cultures was three times higher than when grown on a medium containing 5 ppm - P. Uptake of P by $C_{1,4}$, however, was more than P uptake of $C_{2,1}$. About half of the P used by $C_{1,4}$ appeared as soluble organic P. This was due either to production by $C_{1,4}$ of more soluble extracellular P - containing compounds, or to the release of soluble P - containing compounds after the death of the cells subsequent to the fourth day of incubation as mentioned earlier. Further, the percentage of P in the cells of $C_{1,4}$ was about twice as great as in $C_{2,1}$ when grown in media with 25 ppm P or more.

Biomass -P (Pc) of S_4 reached its peak (under the conditions of this experiment) when the medium was supplied with 65 ppm P (Figure 7). The increase of P concentration in the medium to 105 ppm did not increase biomass - P. When P concentration was further increased to 505 ppm, biomass - P decreased slightly from 1.21 mg P to 0.979 mg P. This, however, was not the case with P uptake. Under the same

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[1] P uptake = Biomass - P (Pc) + soluble organic P (Pso), assuming no inorganic P was released or mineralized after uptake.

Table: 10 Uptake of P by PDB and cell dry weight after 10 days of incubation with 50 ml medium and different concentrations of phosphorus.

Initial Concentration of P (ppm)	Pc (mg) (biomass - P)			Pso (mg) (Organic soluble P)			Po (mg) (P uptake = Pc + Pso)			Dry weight (mg)		
	Cultures			Cultures			Cultures			Cultures		
	S_4	C_{14}	C_{21}	S_4	C_{14}	C_{21}	S_4	C_{14}	C_{21}	S_4	C_{14}	C_{21}
5	-	0.199	0.219	-	0.249	0.125	-	0.448	0.364	-	24.1	30.7
25	0.810	0.585	0.524	0.294	0.725	0.136	1.11	1.31	0.659	-	26.6	53.6
65	1.21	0.577	0.594	1.16	0.656	0.215	2.37	1.23	0.800	-	22.9	58.3
105	1.21	0.655	0.635	1.53	0.624	0.260	2.74	1.29	0.895	-	26.8	56.1
505	0.979	-	-	1.90	-	-	2.88	-	-	-	-	-

All values are expressed as mg P/flask

conditions P uptake by S_4 increased (but not significantly) from 2.74 to 2.88 mg P with the increase of initial P concentration from 105 to 505 ppm (Table 10). On the other hand, production of soluble P - containing compounds increased with the increase of P concentration in the medium.

Some observations about the dynamics of P in these cultures can be made from the data at hand. The peaks of P uptake by C_{14} and C_{21} occurred after two days of incubation (Figures 15 and 16), and by S_4 after four days (figure 17). This was parallel to biomass weight (Table 11). Data about CO_2 evolution and glucose uptake would help to give a comprehensive picture of P uptake, but were not measured. Cole *et al*, (1977) found that the peaks of CO_2 evolution appeared after two days of incubation in soil amended with glucose.

The maximum quantity of P consumed varied with the organisms used, being 2.3 fold greater for S_4 than for C_{14}, and 1.97 times as great as in C_{21} (Table 11).

P, once converted to biomass, did not all remain as such, but was either converted to a soluble organic component or to inorganic P.

The reduction in total organic P after day 2 for culture C_{21} was generally matched by a concomitant increase in Psi (Figure 16). The number of cells also decreased after reaching a maximum of $7*10^{10}$ cells ml^{-1} at day 2 (Figure 10). The increase in inorganic P in solution (Psi) might be

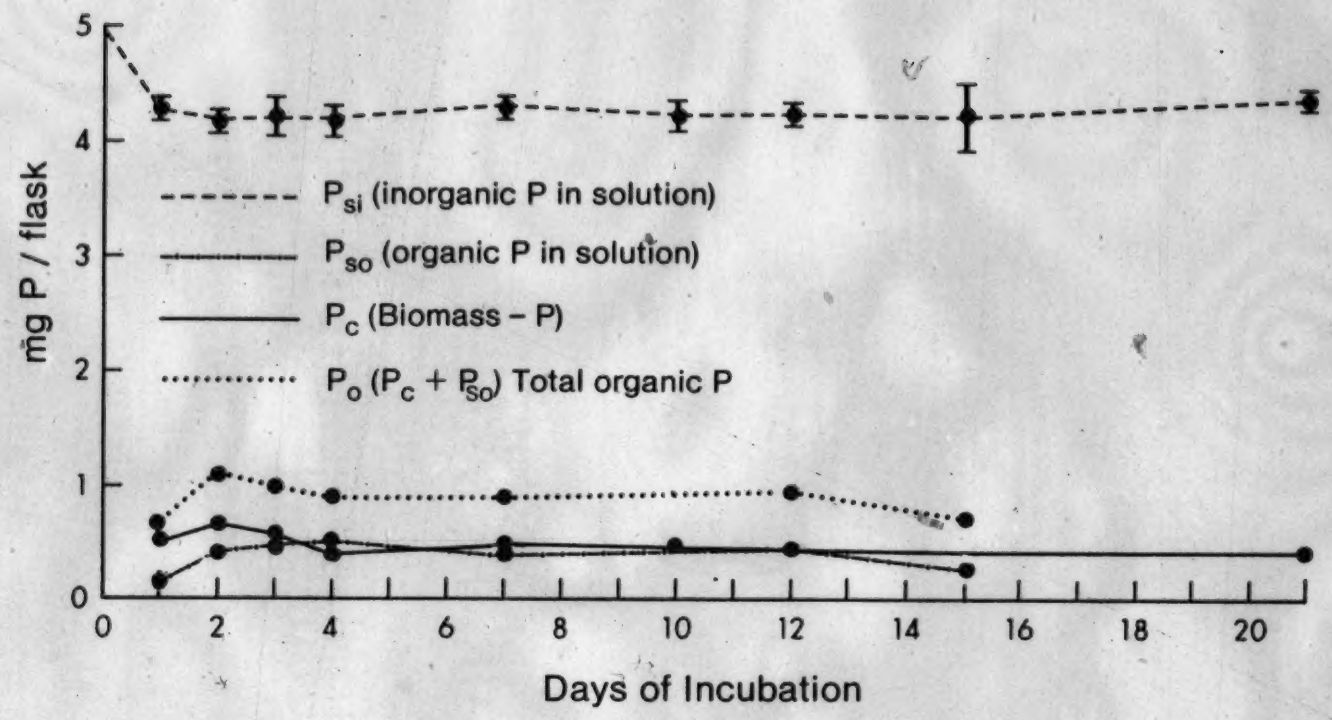

Figure 15: Uptake of P by C_{14}

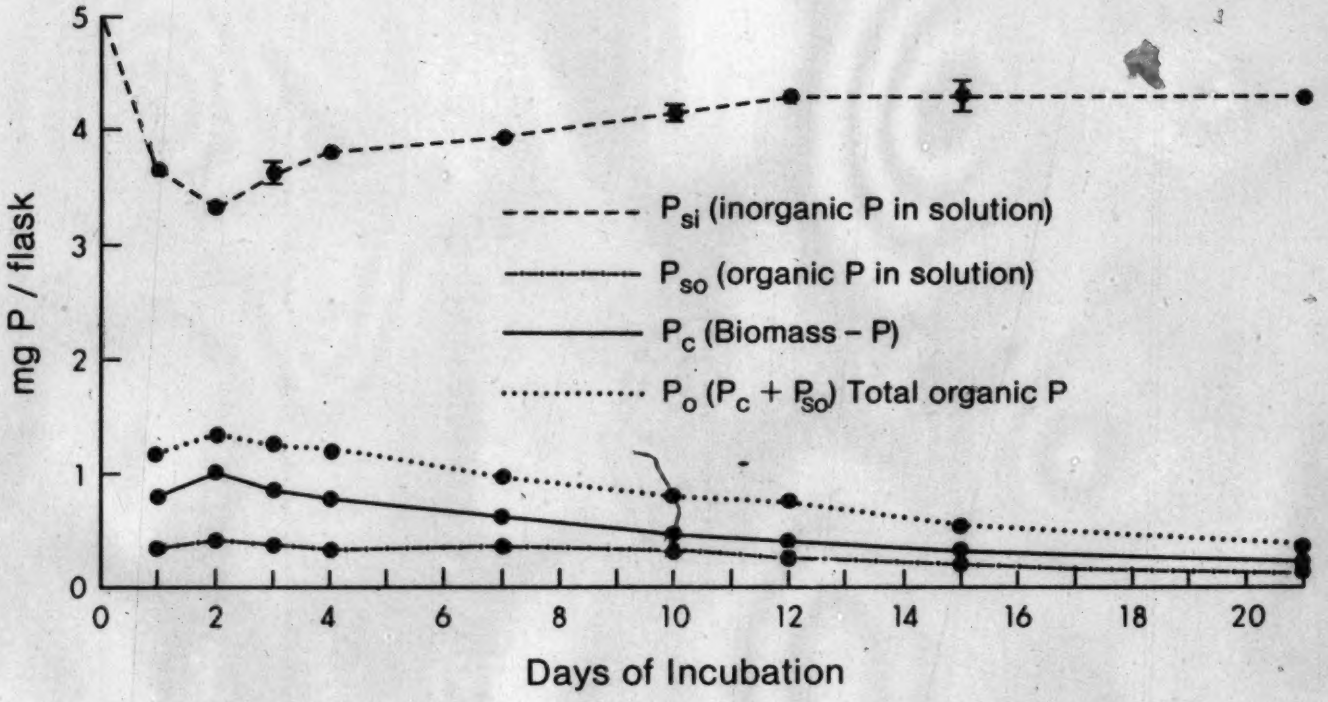

Figure 16: Uptake of P by C_{21}. Only one sample was analyzed for P_{so} after 1, 3, 4, 15 and 21 days.

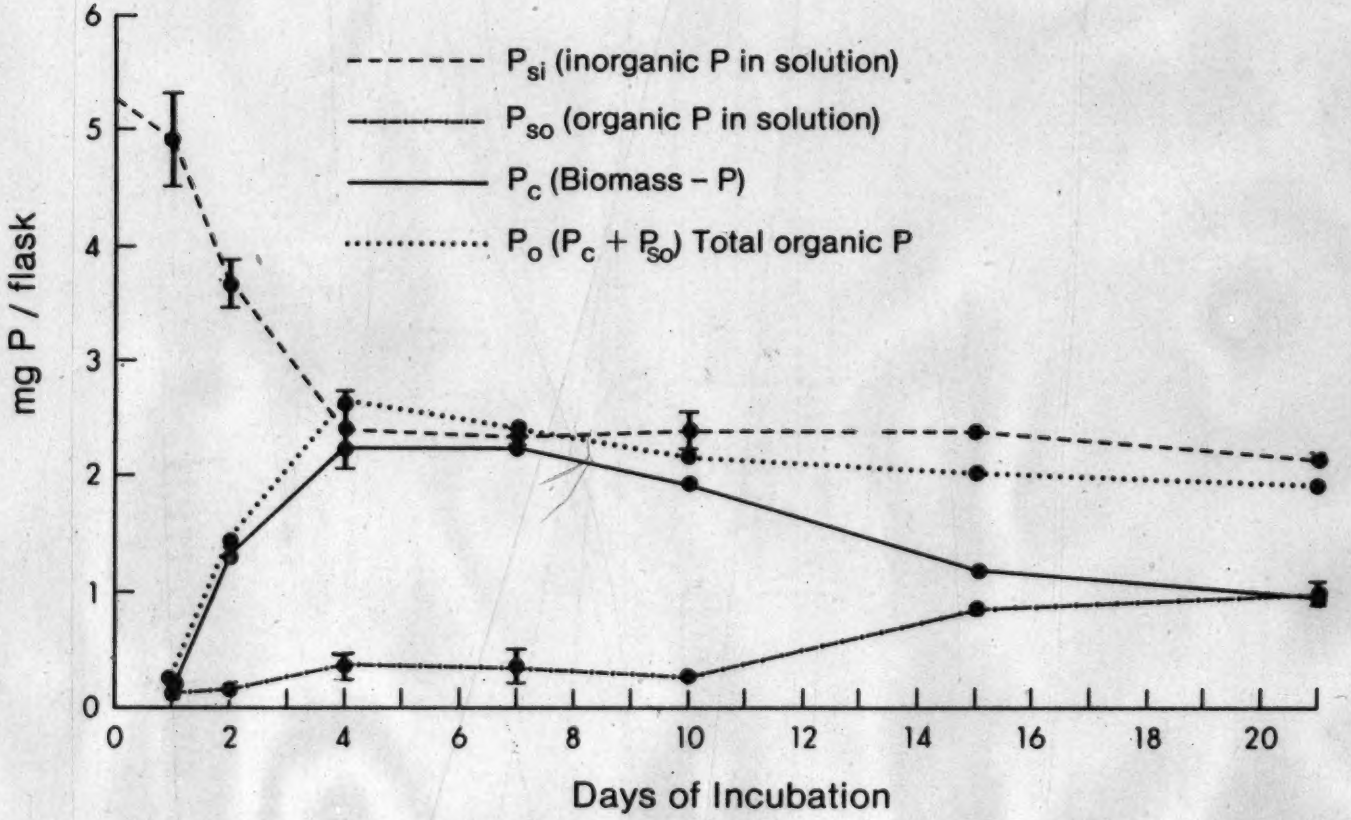

Figure 17: Uptake of P by S_4. Standard deviation of P_{si} value after 15 days is zero.

Table: 11 Uptake of P by PDB and cell dry weight at different times of incubation. Concentration of P in the original medium was 105 ppm and the initial volume was 50 ml.

Days of incubation	Pc (mg) (biomass - P)			Pso (mg) (Organic soluble P)			(Po) mg P uptake = Pc + Pso			Dry weight (mg)		
	Cultures			Cultures			Cultures			Cultures		
	S_4	C_{14}	C_{21}	S_4	C_{14}	C_{21}	S_4	C_{14}	C_{21}	S_4	C_{14}	C_{21}
1	0.149	0.482	0.799	0.115	0.155	0.350	0.264	0.637	1.15	-	21.5	43.4
2	1.35	0.626	0.992	0.140	0.405	0.418	1.49	1.13	1.33	-	29.3	62.4
3	-	0.547	0.851	-	0.443	0.375	-	0.990	1.23	-	26.3	54.8
4	2.27	0.382	0.779	0.360	0.507	0.325	2.63	0.889	1.20	-	24.3	51.4
7	2.06	0.489	0.617	0.340	0.380	0.340	2.40	0.869	0.957	-	-	50.0
10	1.93	0.451	0.471	0.250	-	0.330	2.18	-	0.796	-	-	48.9
12	-	0.435	0.409	-	0.477	0.250	-	0.912	0.759	-	22.5	43.2
15	1.21	0.413	0.333	0.840	0.264	0.215	2.05	0.677	0.548	-	23.4	-
21	0.971	0.418	0.250	0.971	-	0.130	1.94	-	0.380	-	22.6	38.8

All values are expressed as mg P/flask

due either to

1. death and lysis of cells and release of some of the stored polyphosphate, which might hydrolyse under conditions of low pH and show up as Psi,
2. mineralization of the dead cells,
3. both.

Katchman and Van Waza (1974) found that some polyphosphates are soluble and others are insoluble in 5 % cold trichloroacetic acid depending on the degree of polymerization. Harold, (1966) and Elliott *et al* , (in press) found that bacteria consume P in luxury amounts and store it as polyphosphate. Polyphosphate content of bacteria varies according to the growth phase and nutritional conditions (Harold, 1966). Rhee (1973) suggested that polyphosphate may not simply be a reservoir of P, but may be metabolically active, perhaps as a source of stored energy. Therefore, to understand P limitation it is necessary to measure both external P and internal polyphosphate of the cell. Production and activity of phosphatase for organic P mineralization, however, is unlikely because of the high concentration of inorganic P in solution (Spiers and McGill,1979). Further study is needed to clarify this point, but these data do suggest that P, once incorporated into biomass, may be released through cell death even in the absence of predators or other lytic organisms.

Biomass - P and P uptake by C_{14} decreased from 0.626 mg P/flask and 1.13 mg P/flask respectively after two days of

incubation to 0.413 mg P / flask and 0.677 mg P / flask after 15 days incubation (Figure 15). This decrease in biomass corresponded, in part, to an increase in inorganic P in solution. The cells of this organism died after four days. Therefore, the only explanation for the increase in inorganic P is lysis of the cells and the release of P.

As for S_4, biomass - P decreased after four days, but no increase in inorganic P was observed (Figure 17). Instead, the soluble organic P content increased almost by the same amount. This means that this organism converted most of the inorganic P to soluble or insoluble P - containing organic compounds and/or polyphosphate.

The phosphorus content of various microorganisms vary from approximately 2.5 % - 5 % (dry weight) (Porter, 1947). In growing *E. coli* cells the total P measured by ^{32}P tracer was 3.2 % of the dry weight (Luria, 1960). Mitchell and Moyle, (1954) measured P content of various bacteria and found it to vary considerably, even between strains (Table 12). Van Veen *et al* , (1979) reported values ranging from 1.1 to 1.98 %, depending on the organism, media composition and incubation conditions. The results of the present study lie in the range of those found in the literature (Table 12), and vary according to the organism, the growth condition and the period of incubation. (Figures 18 and 19). At a low concentration of P (5 ppm) in the medium and after ten days of incubation, no significant difference was observed between P content of $C_{1,4}$ and $C_{2,1}$ (Figure 18). At

Table 12: P Content of various bacteria

Organism	P content(% of dry wt.)	Culture Condition	Reference
Agrobacterium globiformis	1.93	Shake culture, nutrient broth	Van Veen et al (1979)
" "	1.53	3.25 atm. nutrient broth	" " "
" "	1.98	13.75 atm. " "	" " "
" "	1.12	3.25 atm. soil extract	" " "
" "	1.86	13.75 soil extract	" " "
Enterobacter aerogenes	1.98	Shake culture, nutrient broth	" " "
" "	1.56	3.25 atm. nutrient broth	" " "
" "	1.45	13.75 atm. " "	" " "
" "	1.53	3.25 atm. soil extract	" " "
" "	1.11	13.75 atm. soil extract	" " "
Mycobacterium thamnopheos	2.62	Synthetic medium, 24 hours	Mudd et al. (1958)
" "	2.51	Dubose + glucose, 24 hours	" " "
Mycobacterium smegmatis	0.88	P & B + Fe^{+++} medium, 2 days	Winder & Denney (1956)
" "	2.02	" " 7 days	" " "

* Calculated from the data of Mudd et al. (1958) assuming that each 12 mg dry weight contain one mg nitrogen (Winder & Denney, 1956)

... continued

Organism	P content (% of dry wt.)	Culture Condition	Reference
Corynebacterium diphtheriae [E]	2.17	after 10 minutes	Sall et al (1958)
" "	2.82	" 20 "	" " "
" "	1.68	" 30 "	" " "
" "	2.24	" 40 "	" " "
" "	1.36	" 50 "	" " "
" "	1.75	" 60 "	" " "
E. Coli	3.20	measured by P^{32} tracer	Luria (1960)
Pseudomonas paucimobilis [*]	2.62	sterilized soil amended with 700 ug glucose-c g^{-1} and 81 ug NH^{4+} - NG^{-1} and incubated for 63.5 hr.	Elliott et al (in Press)
" "	2.08	" " " " 39.5 h.	" " "
" "	2.83	" " " " 15.5 h.	" " "
E. Coli	2.87		Leach (1906) as cited by Porter (1947)
Azotobacter chroococcum	3.94		Stoklasa (1908) as cited by Porter (1947)
Staphylococcus bacteriophage	4.6 - 5.0		Northrop (1938) as cited by Porter (1947)

. . . continued

[E] Calculated from the data of Sall et al. (1958) assuming that the cells have a dry weight of 3.24×10^9 cells/mg (Palmer & Mallette, 1961)

[*] Calculated from the data of Elliott et al. assuming that 44% (the average of 12 bacteria) of the dry weight of bacteria is carbon. (Anderson & Domsch, 1978)

Organism	P content (% of dry wt.)	Culture Condition	Reference
Aerobacter aerogenes	1.84	Grown on a casein digested medium containing 1% glucose and 0.1% Marmite and harvested during the phase of decelerated growth	Mitchell & Moyle (1954)
Azotobacter agile	2.17		" " "
Bacillus brevis	1.79		" " "
Bacillus subtilis	3.59		" " "
E. Coli (H)	2.06		" " "
E. Coli (Ms)	1.45		" " "
Proteus vulgaris	1.94		" " "
Serratia marcescens	1.89		" " "
Clostridium sporogenes	2.40		" " "
Clostridium welchii	2.97		" " "
Staphylococcus aureus	2.59		" " "
Streptococcus faecalis	3.25		" " "
Coryrnbacterium xerosis	3.03		" " "

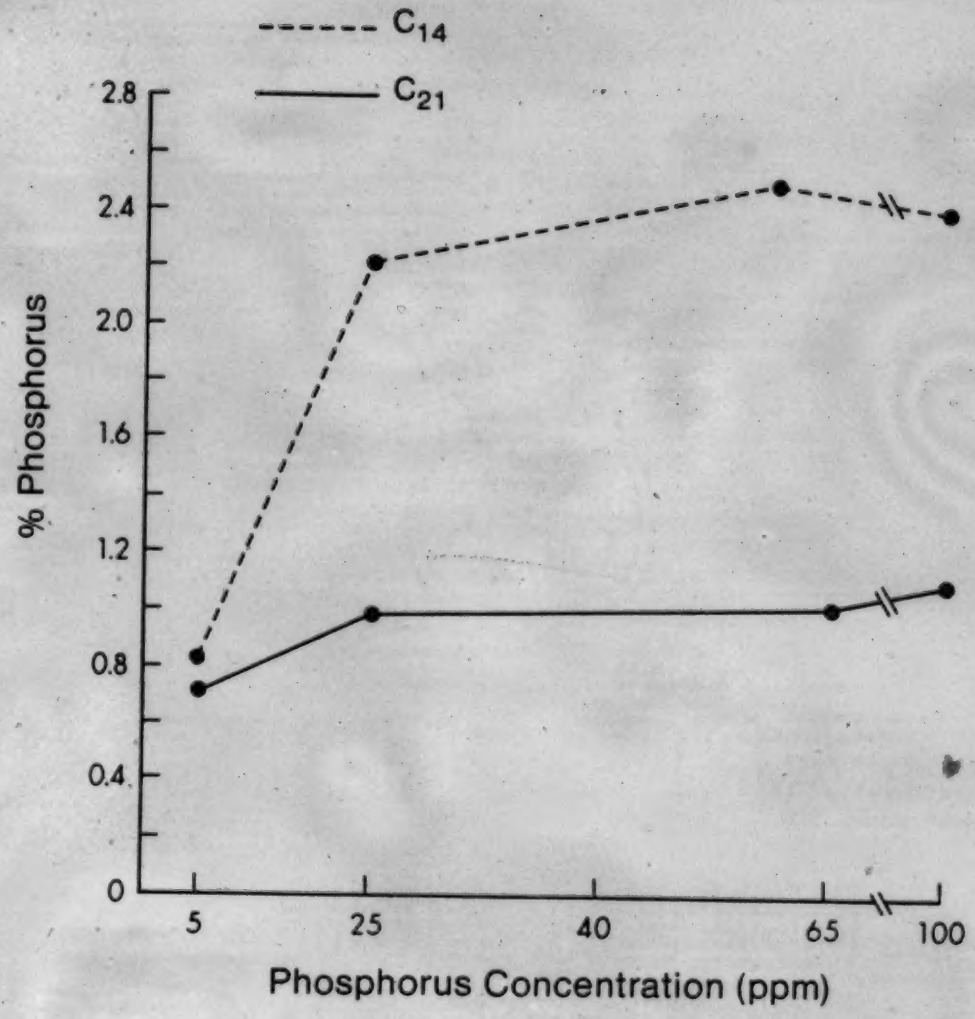

Figure 18: Phosphorus content of bacteria after 10 days of incubation with different initial concentrations of P in the medium.

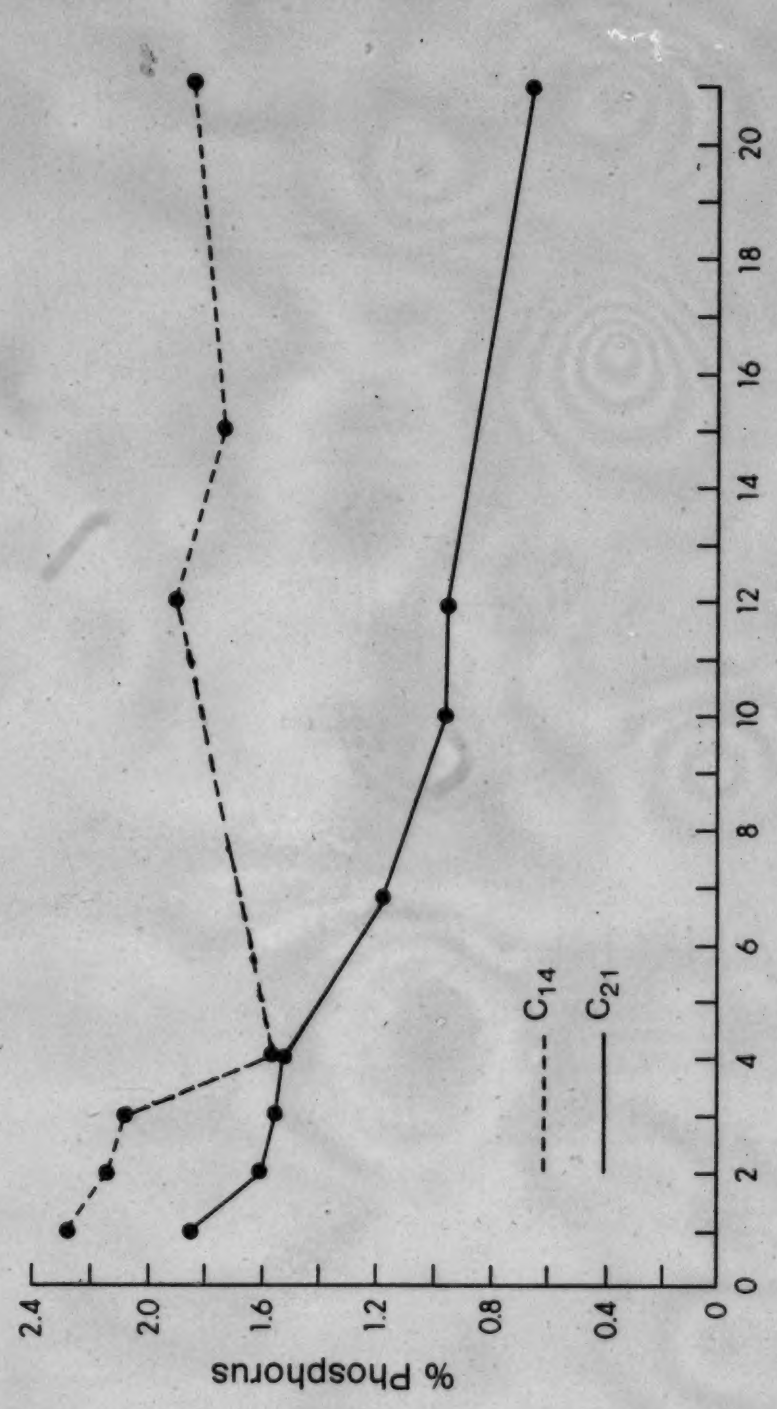

Figure 19. Phosphorus content of bacteria after different incubation periods. Concentration of P in the medium was 105 ppm.

high concentrations of P, however, the P content of C_{14} was twice as much as that of C_{21} (1.1% compared to 2.5%). In both C_{14} and C_{21} the percentage of P (dry basis) reached its maximum within one day of incubation (Figure 19). Sall *et al* (1958) measured total P in bacterial cells over a period of one hour and found that the maximum accumulation of P in the cell occurred after 20 minutes ($8.7*10^{-9}$ gm P/Cell). Rhee, (1973), Harold, (1966), Mudd *et al*, (1958) and Sall *et al* , (1958) found that peaks of polyphosphate accumulation occurred immediately before the exponential phase; minimal amounts of polyphosphate were found at the end of the stationary phase.

5.4 Solubilization of $Ca_{10}(OH)_2(PO_4)_6$ by Several Phosphate Dissolving Bacteria (PDB) in Buffered and Unbuffered Systems

5.4.1 Introduction

It is well established that a group of soil microorganisms can bring the phosphorus of sparingly soluble phosphate minerals into solution in an unbuffered liquid medium (Banik and Dey, 1981; Hayman, 1975; Agnihotri, 1970; Chhonkar and Subba - Rao, 1965; Sperber, 1958; Swaby and Sperber, 1959 and Rose, 1956). In buffered systems, however, the situation might not be the same. Swaby and Sperber (1959), and Ahmed and Jha (1968) found that the addition of calcium carbonate to a culture medium of PDB caused a

drastic decrease in the amount of orthophosphate brought into solution from sparingly soluble phosphate minerals. They concluded that microbial solubilization of phosphate minerals is unlikely in neutral to alkaline soils. Because the buffering capacity of sandy soils is low, it is possible that in such soils rock phosphate may be dissolved by PDB (Swaby and Sperber, 1958).

Banik and Dey (1981) and Sundara - Rao *et al*,(1963) reported that PDB had the ability to increase the available phosphorus in soils (pH 6.8 or 5.4) supplied with rock phosphate. The dry weight of lavender grown in calcareous soil inoculated with phosphate dissolving bacteria (PDB) increased steadily with rock phosphate concentration (Azcon *et al*, 1976).

Banik and Day (1981) suggested that the presence of soluble phosphate in the medium might restrain the organisms from utilization of the insoluble phosphate. They also related the highest solubilization by *Aspergillus niger* and *Streptomyces sp.* during the sporulation period to the highest demand of P during that period. Their conclusion implies that solubilization of rock phosphate is controlled by the need for phosphorus. They did not, however, provide any direct evidence for this conclusion. In contrast to the finding of Banik and Dey, Chhonkar and Subba - Rao (1967) found that *Aspergillus niger* solubilized phosphate minerals in both the presence and the absence of soluble phosphorus.

In all the previous studies, the amount of orthophosphate released by phosphate dissolving microorganism in solution was considered as the only parameter by which to assess their capacity to dissolve phosphate minerals. No attempt was made, either in soil or in the laboratory, to evaluate the ability of these microorganisms to convert the insoluble phosphate minerals into organic phosphate.

The following experiments were designed to :

1. Examine the partitioning between biomass, soluble organic and soluble inorganic P, of P removed from $Ca_{10}(OH)_2(PO_4)_6$ during growth of PDB in an unbuffered system.
2. Examine the ability of cultures C_{21} and C_{14} to dissolve $Ca_{10}(OH)_2(PO_4)_6$ in a system buffered with $CaCO_3$.

5.5 Materials and Methods.

> 1. Partitioning of P dissolved from $Ca_{10}(OH)_2(PO_4)_6$ in an unbuffered system.

Several previously screened cultures of PDB were used. All analytical methods, media and incubation conditions were as previously described. The HCl method was used to separate cells from residual $Ca_{10}(OH)_2(PO_4)_6$ particles.

2. Partitioning of P dissolved from $Ca_{10}(OH)_2(PO_4)_6$ in a buffered system.

Forty four ml of the previously described liquid medium were dispensed into each 125 ml erlenmeyer flask containing 50 mg $Ca_{10}(OH)_2(PO_4)_6$. The flasks were then divided into groups of four, with each group receiving different amounts of $CaCO_3$ (0.0, 25, 50 and 75 mg $CaCO_3$). To counteract the change in pH caused by hydrolysis of $CaCO_3$ during autoclaving, the pH of the medium was adjusted to 8.9, 7.8, 7.4 and 7.4 for the four treatments respectively on the basis of a preliminary examination of the change in pH during autoclaving. The flasks were then autoclaved at 120°C and 103 kPa for 40 minutes. After autoclaving the pH was checked in 4 flasks of each treatment and was found to be 8.0, 7.9, 8.0 and 8.0 for the four treatments respectively. The flasks were then inoculated and incubated as previously described. Each treatment was replicated four times. After 10 days of incubation, the flasks were removed and Psi, Pso and Pc were measured as previously described.

5.6 Results and Discussion.

1. Dissolution of HA in an unbuffered system.

The initial pH of the cultures before incubation was 7.1. After ten days of incubation pH of the control was 6.8. Organism S_4 and C_{21} raised the pH of the culture to 8.4 and 7.8 respectively. The other organisms lowered the pH to between 3.1 and 4.9 (Figure 20). As reported previously (Figure 11), cultures C_{21} and S_4 did not increase the pH

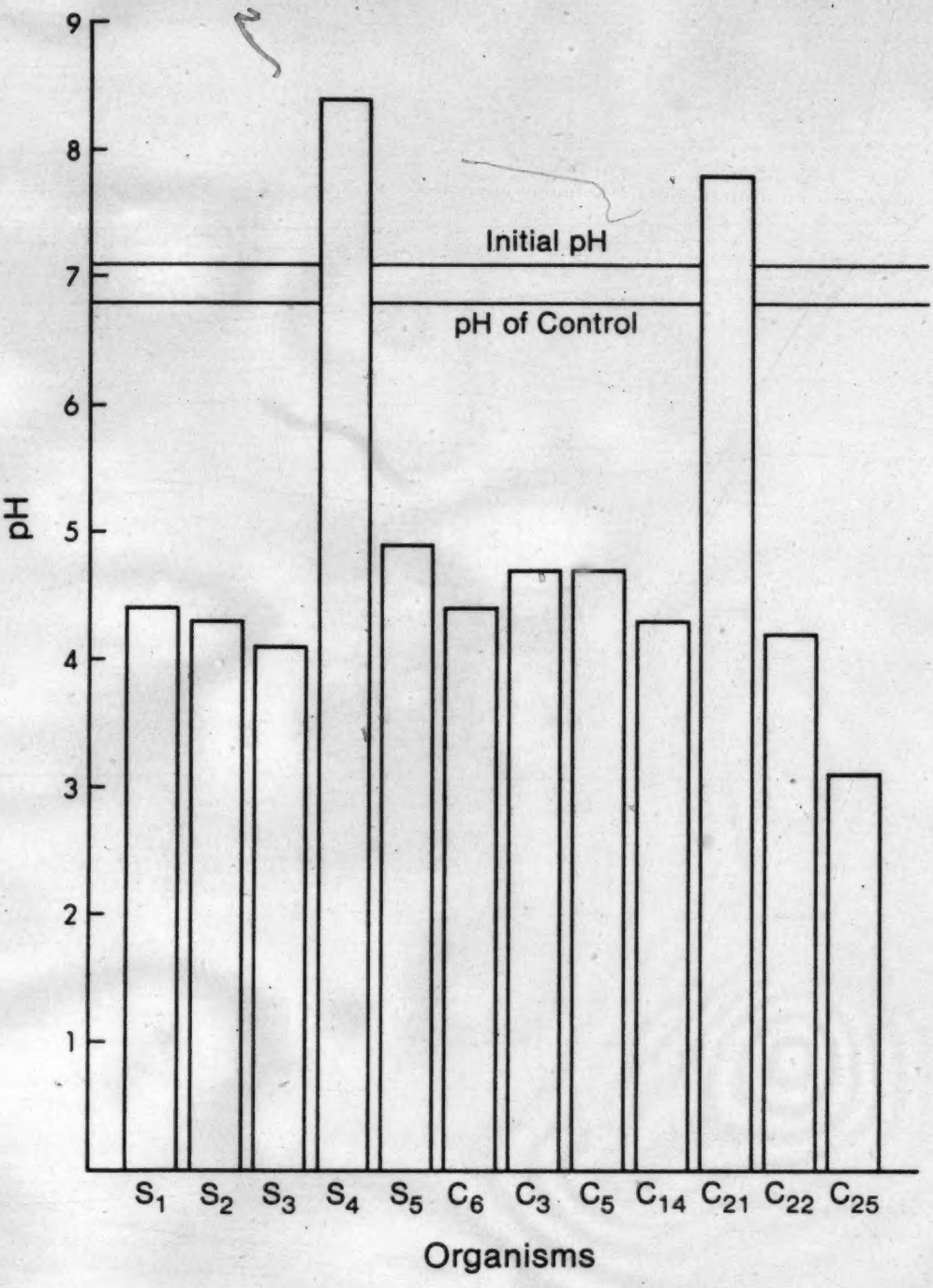

Figure 20. pH of several phosphate dissolving bacterial cultures after 10 days of incubation with 50 mg $Ca_{10}(OH)_2(PO_4)_6$/50 ml medium.

continuously during the period of incubation. Goswani and Abhiswas, (1962); Das, (1963); Chhonkar and Subba - Rao, (1967) and Ahmad and Jha, (1968) reported a similar increase in pH by some PDB at the end of incubation period.

The group of organisms which lowered the pH converted between 6 and 30 % of the total P dissolved into cell materials. The group that raised the pH converted between 80 - 88% of the total dissolved P into cell materials, but dissolved less total P than did the former group (Figure 21). Microbial P (Po) to orthophosphate ratio was 4 and 7 for the cultures C_{21} and S_4 respectively, and was less than 0.4 for the rest of the cultures (Figure 22).

The equilibrium dissociation of $Ca_{10}(OH)_2(PO_4)_6$ can be given by the following reaction (Lindsay, 1979) :

$Ca_{10}(OH)_2(PO_4)_6$ ..... $10Ca^{2+} + 6PO_4^{3-} + 2OH^-$.

In the present system, equilibrium is hard to establish because of :

1. The partial removal of PO_4^{3-} by bacterial cells.
2. The release of some PO_4^{3-} from dead cells.
3. The possible production of organic anions having the ability to chelate Ca^{2+}, thus removing it from the aqueous system.

It can be seen from the above equation that lowering pH shifts the equilibrium to the right, thus increasing solubility of $Ca_{10}(OH)_2(PO_4)_6$. Consequently, HA is unstable under acidic conditions. Also, removal of Ca^{2+} from the system by chelating compounds would increase solubilization.

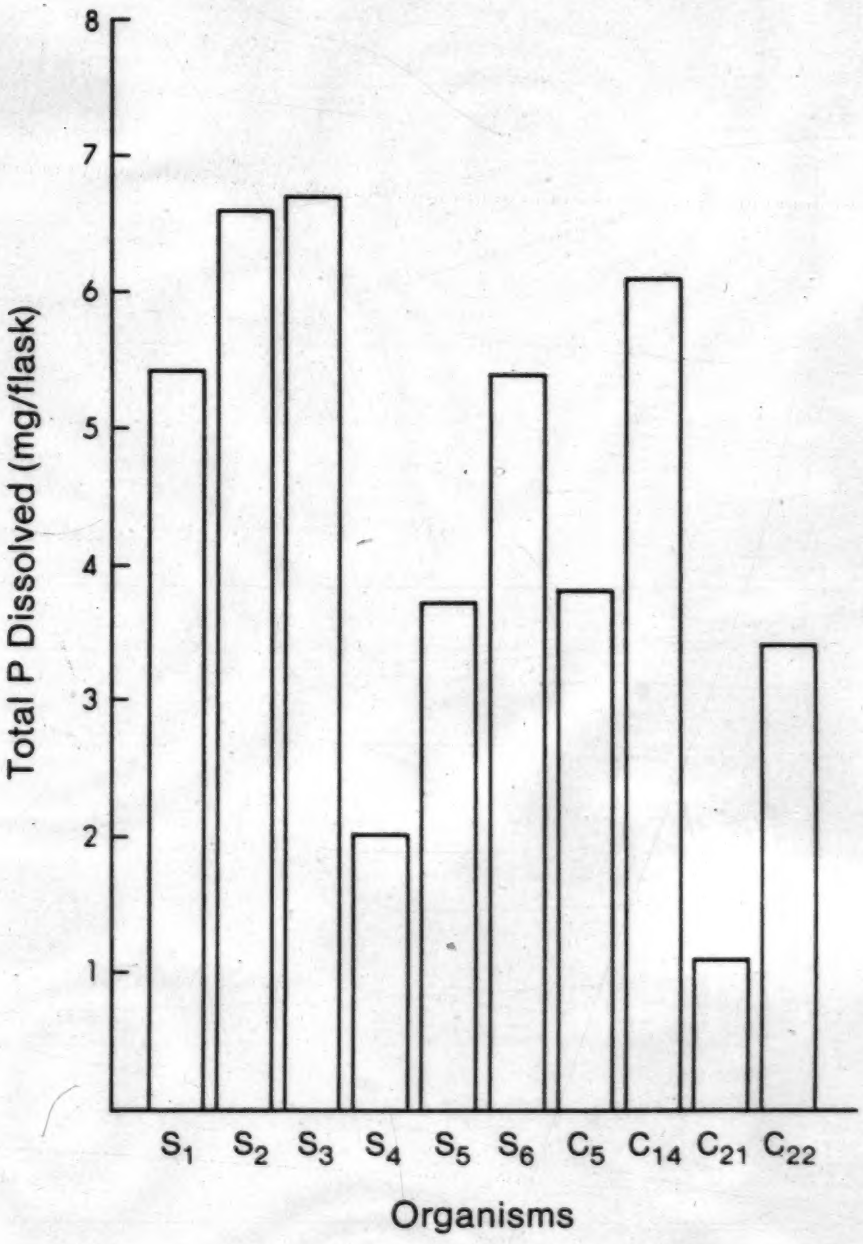

Figure 21. Total phosphorus dissolved following 10 days of incubation of several phosphate dissolving bacteria with 50 mg $Ca_{10}(OH)_2(PO_4)_6$/50 ml medium.

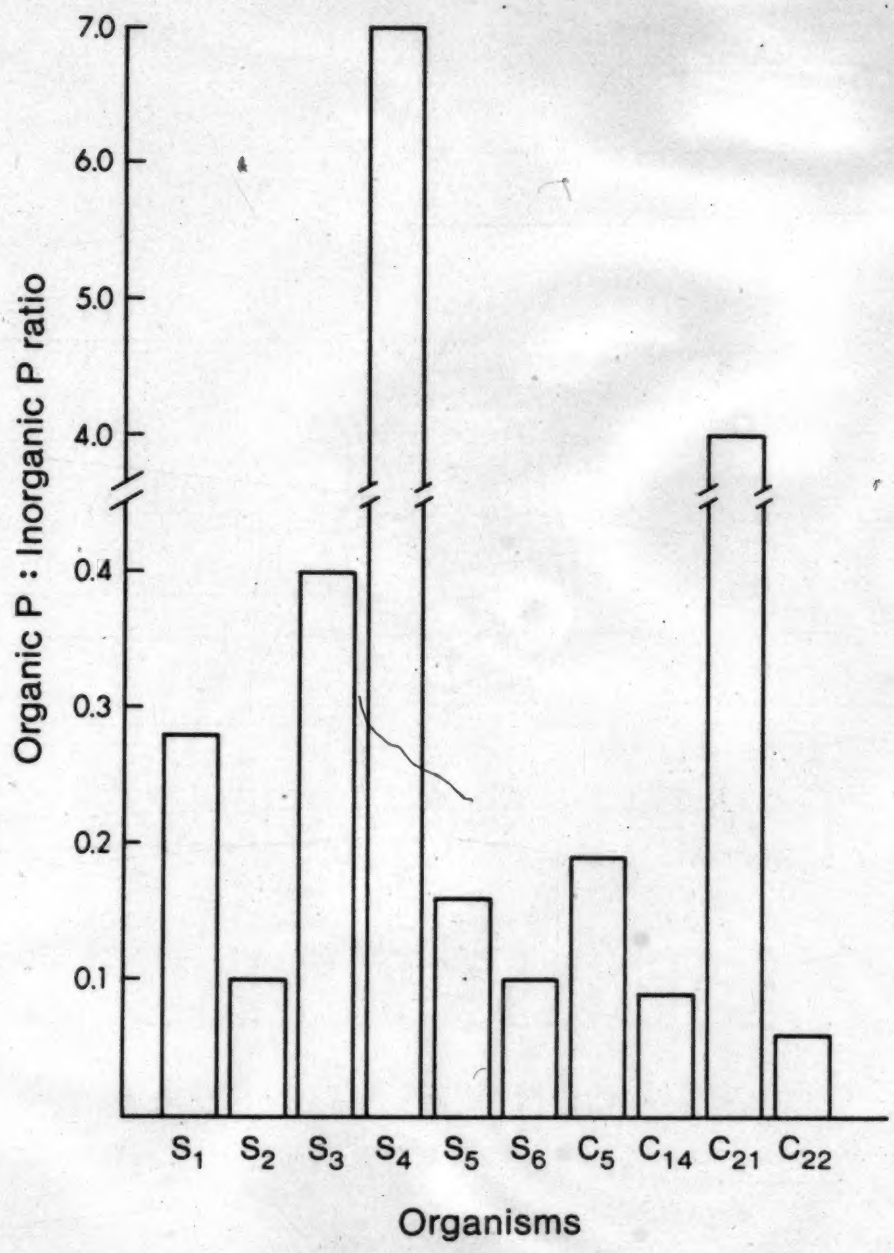

Figure 22. Organic P: Inorganic P in solution ratio of several phosphate dissolving bacteria after 10 days of incubation with 50 mg $Ca_{10}(OH)_2(PO_4)_6$/50 ml medium.

There was a close inverse correlation between $\ln[H_2PO_4^-]$ and final pH ($r^2 = 0.94$; ($p=0.01$), Figure 23). Data from the literature, summarized in Table 13, also show a highly significant inverse relation ship between final pH and $\ln[H_2PO_4^-]$ in solution. The above results suggest that, under the conditions of this experiment and for the specific organisms tested, pH was the main factor controlling variation in concentration of P in solution.

Lindsay (1979) plotted solubility of $Ca_{10}(OH)_2(PO_4)_6$ against pH (Figure 24). The activity of Ca used to develop this diagram was $10^{-3.5}$M or fixed by $CaCO_3$ (calcite) and CO_2(g) at 0.0003 atmospheres. In general, solubilization of HA in our system does not follow the line of HA solubility in Lindsay's (1979) diagram. This may be because of one or more of the following :

1. Activity of Ca^{2+} in this system may be different from that used in the relation in Lindsay's diagram. Activity of Ca^{2+} in the present system is unknown and very hard to calculate because of the heterogeneity of the system.
2. Removal of some of the solubilized P by bacterial cells.
3. Factors other than pH control solubilization of HA.

The correlation between pH and the total amount of P dissolved ($r^2 = 0.36$) was very much lower than that between pH and orthophosphate concentration in solution, reflecting , in part, the independent effect of P incorporation into biomass. For example, the final medium pH produced by culture C_{22} was lower than that produced by cultures S_{11}, S_{21},

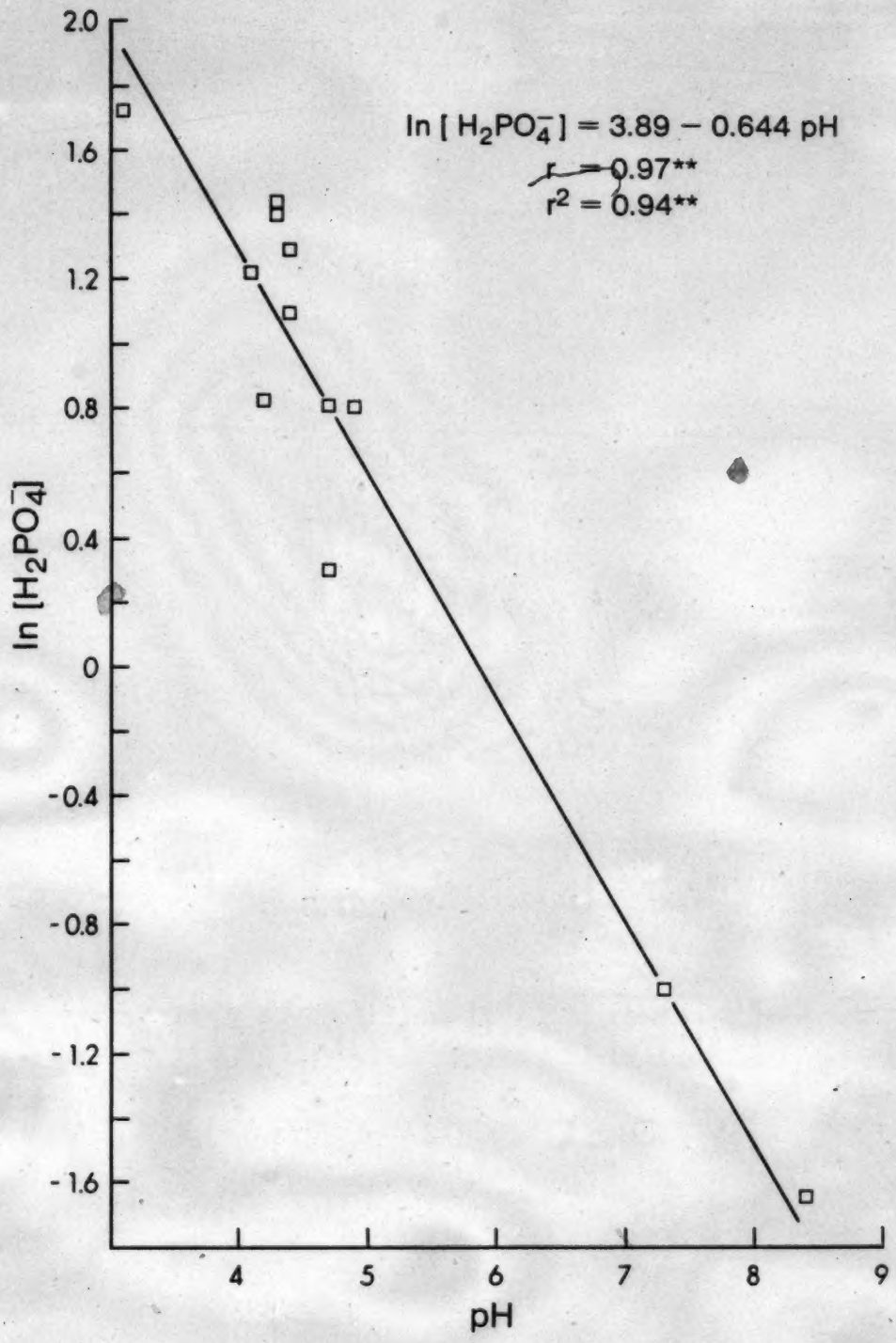

Figure 23. Relation between pH and ln [$H_2PO_4^-$] in solution.

Table 13: Relation between pH and ln $[H_2PO^-_4]$ in Solution

Organisms	Correlation Coefficient (r)	Source +
Bacteria	-0.80 **	Paul and Sundara Rao (1971)
Fungi	-0.62 *	Agrihotri (1970)
Fungi	-0.75 **	Chhonkar and Subba Rao (1967)
Bacteria	-0.97 **	(Present Study)

+ I used their data to calculate (r)
* Significant (p=0.05)
** Significant (p=0.01)

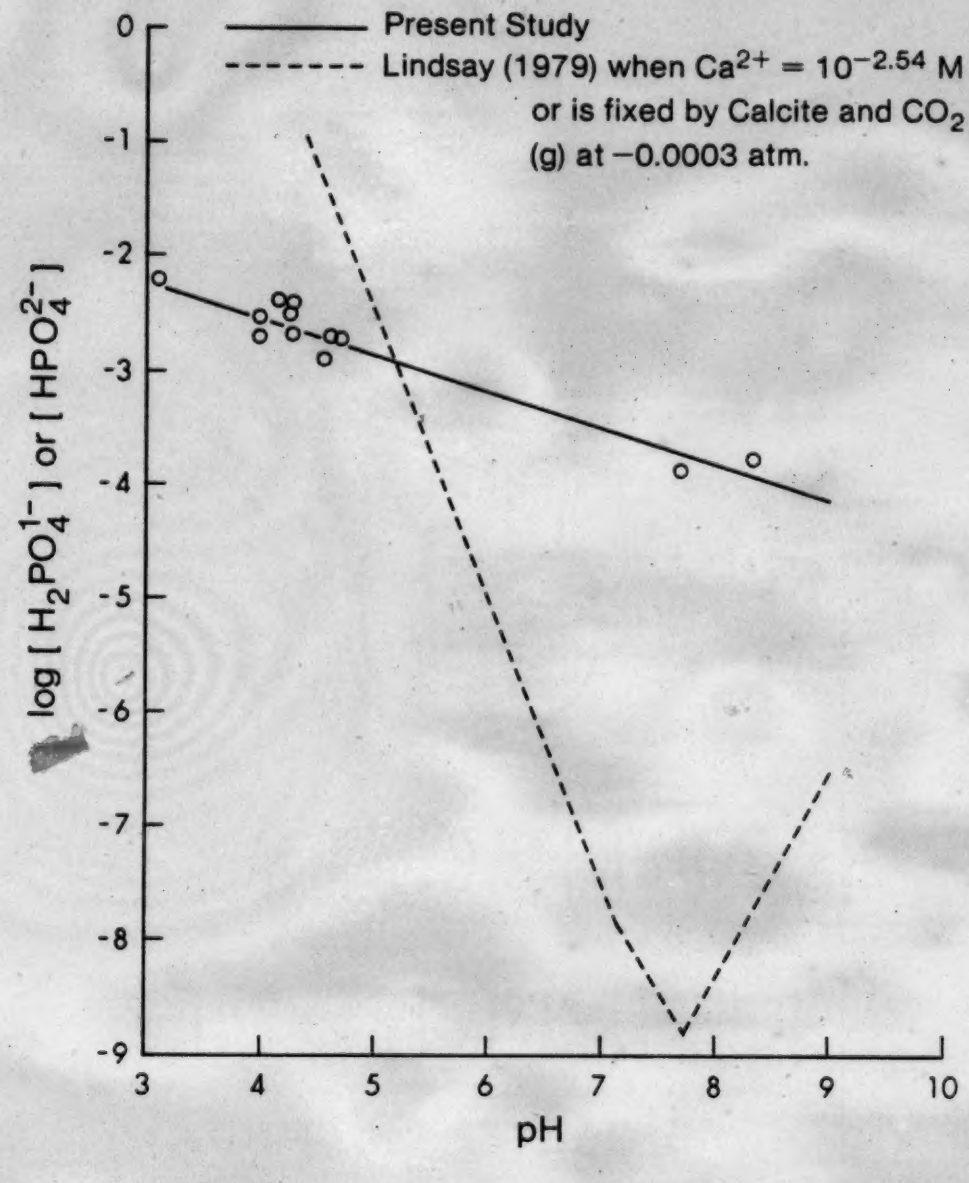

Figure 24. Solubility of $Ca_{10}(OH)_2(PO_4)_6$ as a function of pH.

S_4 and C_{14}, but C_{22} dissolved only about half as much P as did these cultures.

The effect of soluble P on the ability of cultures C_{21} and C_{14} to transform $Ca_{10}(OH)_2(PO_4)_6$ was examined by adding 105 ppm of soluble P (added as K_2HPO_4) to the media. The added P had no effect on the ability of these cultures to transform $Ca_{10}(OH)_2(PO_4)_6$, (Table 14), but it had a slight effect on their capacity to dissolve HA. Culture C_{21} dissolved more P from $Ca_{10}(OH)_2(PO_4)_6$ in the presence of soluble P in the medium, and converted more P into microbial cells. This was probably because of the increased readily available P. The added P kept the pH at 7.3 rather than 8.0 without it. In contrast, C_{14} solubilized less P in the presence of soluble P and converted less P into microbial cells than it did when the sole source of P was hydroxylapatite (Table 14). Chhonakar and Subba - Rao (1967) found that *Curvularia lunata* lowered pH of the medium to 4.0 and consumed all the available P when supplied with soluble P (5g KH_2PO_4/L), but brought no orthophosphate into solution from $Ca_3(PO_4)_2$. This indicated that demand by *Curvularia lunata* for P is very high. Failure to increase orthophosphate in solution from an insoluble source by such organisms is not an indication of their failure to utilize insoluble phosphate. They might transform a large amount of P into biomass but fail to increase orthophosphate in solution. This clearly demonstrates the importance of microbial P when an assessment of the ability of these

Table: 14 Effect of soluble phosphorus on solubilization of $Ca_{10}(OH)_2(PO_4)_6$ by cultures C_{21} and C_{14} after 10 days of incubation with 50 mg $Ca_{10}(OH)_2(PO_4)_6$. Original volume of the medium was 50 ml. All values are expressed as mg P/flask. The data represent the net difference after subtracting the added soluble P.

Treatment	Culture	pH	Psi	Pso	Pc	Po	%P	PT
with 105 ppm soluble P	C21	7.3 ± 0.16	0.390 ± 0.014	0.490	0.561 ± 0.013	1.05	17	1.74
	C14	4.3 ± 0.00	4.74 ± 0.19	0.190	0.215 ± 0.010	0.305	50	5.05
without adding any soluble P	C21	8.0 ± 0.05	0.253 ± 0.01	0.224 ± 0.03	0.386 ± 0.007	0.630	11	1.18
	C14	4.4 ± 0.05	5.53 ± 0.02	0.221 ± 0.02	0.450 ± 0.020	0.671	63	6.20

Psi = Soluble-inorganic P
Pso = Soluble organic P
Pc = Biomass - P (P in cells)
Po = Organic P (Pso + Pc)
%P = % P solubilized
PT = total P solubilized

organisms to utilize or transform P is to be made.

2. Dissolution of $Ca_{10}(OH)_2(PO_4)_6$ in a buffered system.

Calcium carbonate was used as a buffer because:

1. $CaCO_3$ is a common constituent of many soils and
2. it is easy to separate the undissolved $CaCO_3$ from bacterial cells using the HCl method.

Organisms C_{21} and C_{14} were used in this study.

Addition of 75 mg $CaCO_3$/50 ml medium was insufficient to buffer the medium of culture C_{14} in which the added $CaCO_3$ could not maintain the pH above 5 (Table 15,Figure 25). Despite the low pH, $CaCO_3$ caused a drastic decrease in the amount of orthophosphate brought into solution from $Ca_{10}(OH)_2(PO_4)_6$, reducing Psi from 4.69 mg to 0.062 mg (Figure 26). $CaCO_3$, however, did not cause a significant decrease in the amount of P consumed by cells of C_{14} (Figure 26). $CaCO_3$ also decreased the amount of orthophosphate in solution in the medium following growth of culture C_{21} from 0.28 mg to 0.014 mg, but its effect on total P transformed was less (Table 16 and Figure 27).

In calcareous soil or in a system buffered with $CaCO_3$, $Ca_{10}(OH)_2(PO_4)_6$ cannot compete with $CaCO_3$ for H^+ ions. In other words, $CaCO_3$ will dissolve first because the solubility product of $Ca_{10}(OH)_2(PO_4)_6$ ($Ksp = 10^{-113.7}$) is negligible compared to the solubility product of $CaCO_3$ ($Ksp = 10^{-8.4}$) (Lindsay, 1979). Organism C_{14} dissolved all the $CaCO_3$ added to the system (Table 17), while organism C_{21} dissolved only 92 %, 40 % and 27 % of the added 25, 50 and

Table: 15 Effect of $CaCO_3$ on solubilization of $Ca_{10}(OH)_2(PO_4)_6$ by Culture C_{14} after 10 days of incubation with 50 mg $Ca_{10}(OH)_2(PO_4)_6$ /50 ml medium.

Weight of $CaCO_3$ added	pH	Psi	Pso	Pc	Po	ΣP	PT
0.0 mg	4.4 ± 0.03	4.69 ± 0.075	0.73 ± 0.160	0.480 ± 0.012	1.21	63.7	5.90
25 mg	4.8 ± 0.12	2.07 ± 0.037	0.682 ± 0.110	0.500 ± 0.140	1.18	35.1	3.25
50 mg	4.8 ± 0.05	0.552 ± 0.025	0.413 ± 0.085	0.594 ± 0.032	1.01	16.9	1.56
75 mg	5.0 ± 0.06	0.062 ± 0.005	0.442 ± 0.050	0.436 ± 0.018	0.878	14.2	0.940

Psi, Pso, Pc, Po, ΣP and PT as defined in Table 14

All values are expressed as mg P/flask.

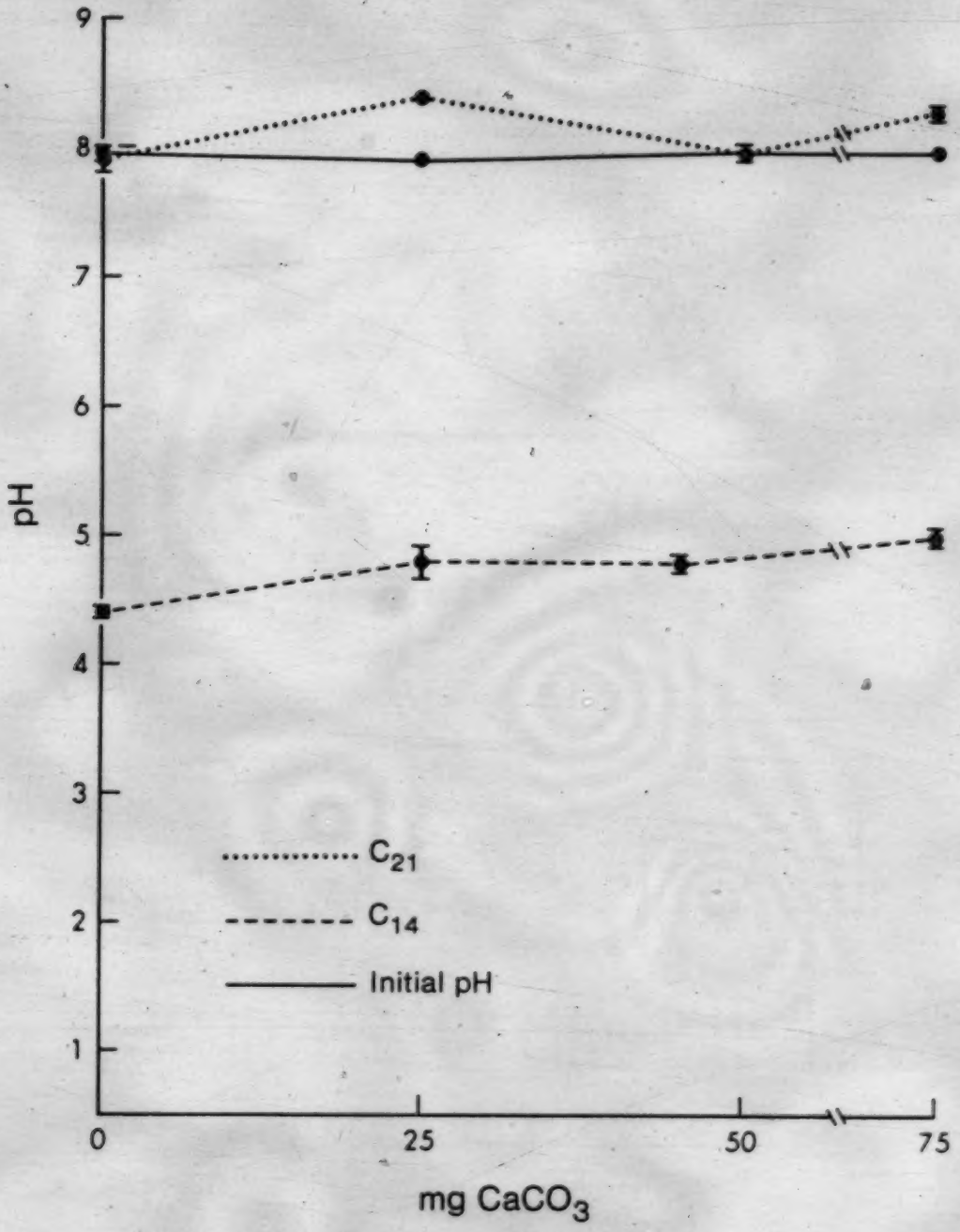

Figure 25. Effect of $CaCO_3$ on pH of cultures C_{21} and C_{14} after 10 days incubation with 50 mg $Ca_{10}(OH)_2(PO_4)_6$ /50 ml medium. Standard deviation of pH value at 25 mg $CaCO_3$ is zero.

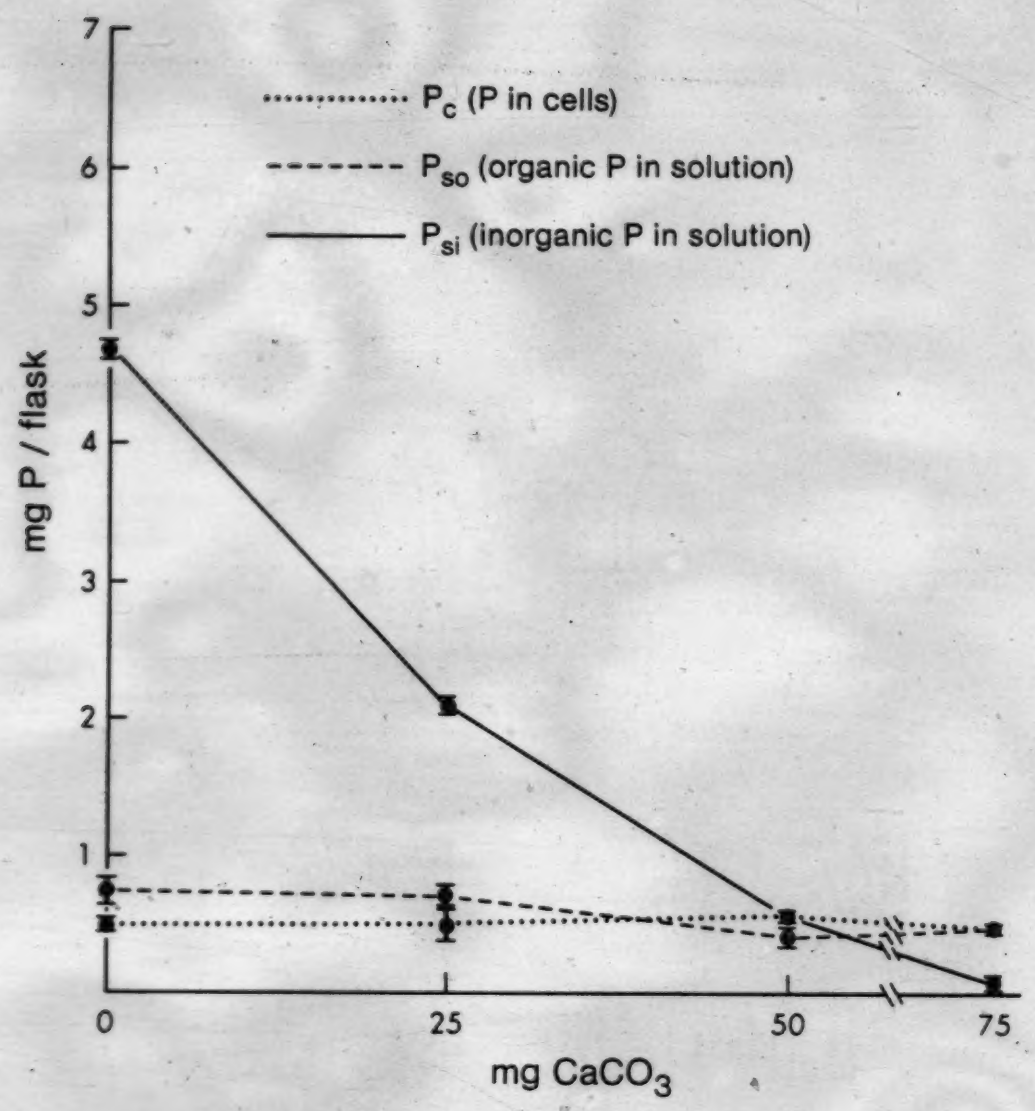

Figure 26. Effect of $CaCO_3$ on solubilization of $Ca_{10}(OH)_2(PO_4)_6$ by culute C_{14} after 10 days of incubation with 50 mg $Ca_{10}(OH)_2(PO_4)_6$/50 ml medium.

Table: 16 Effect of $CaCO_3$ on solubilization of $Ca_{10}(OH)_2(PO_4)_6$ by Culture C_{21} after 10 days of incubation with 50 mg $Ca_{10}(OH)_2(PO_4)_6$ /50 ml medium.

Weight of $CaCO_3$ added	pH	Psi	Pso	Pc	Po	ΣP	PT
0.0 mg	7.9 ± 0.10	0.284 ± 0.030	0.295 ± 0.019	0.511 ± 0.035	0.806	12	1.09
25 mg	8.4 ± 0.00	0.087 ± 0.020	0.206 ± 0.009	0.300 ± 0.011	0.406	5.3	0.593
50 mg	8.0 ± 0.01	0.031 ± 0.004	0.116 ± 0.017	0.277 ± 0.005	0.443	5.1	0.474
75 mg	8.3 ± 0.06	0.014 ± 0.001	0.125 ± 0.012	0.224 ± 0.015	0.362	4.1	0.376

Psi, Pso, Pc, Po, ΣP and PT as defined in Table 14

All values are expressed as mg P/flask.

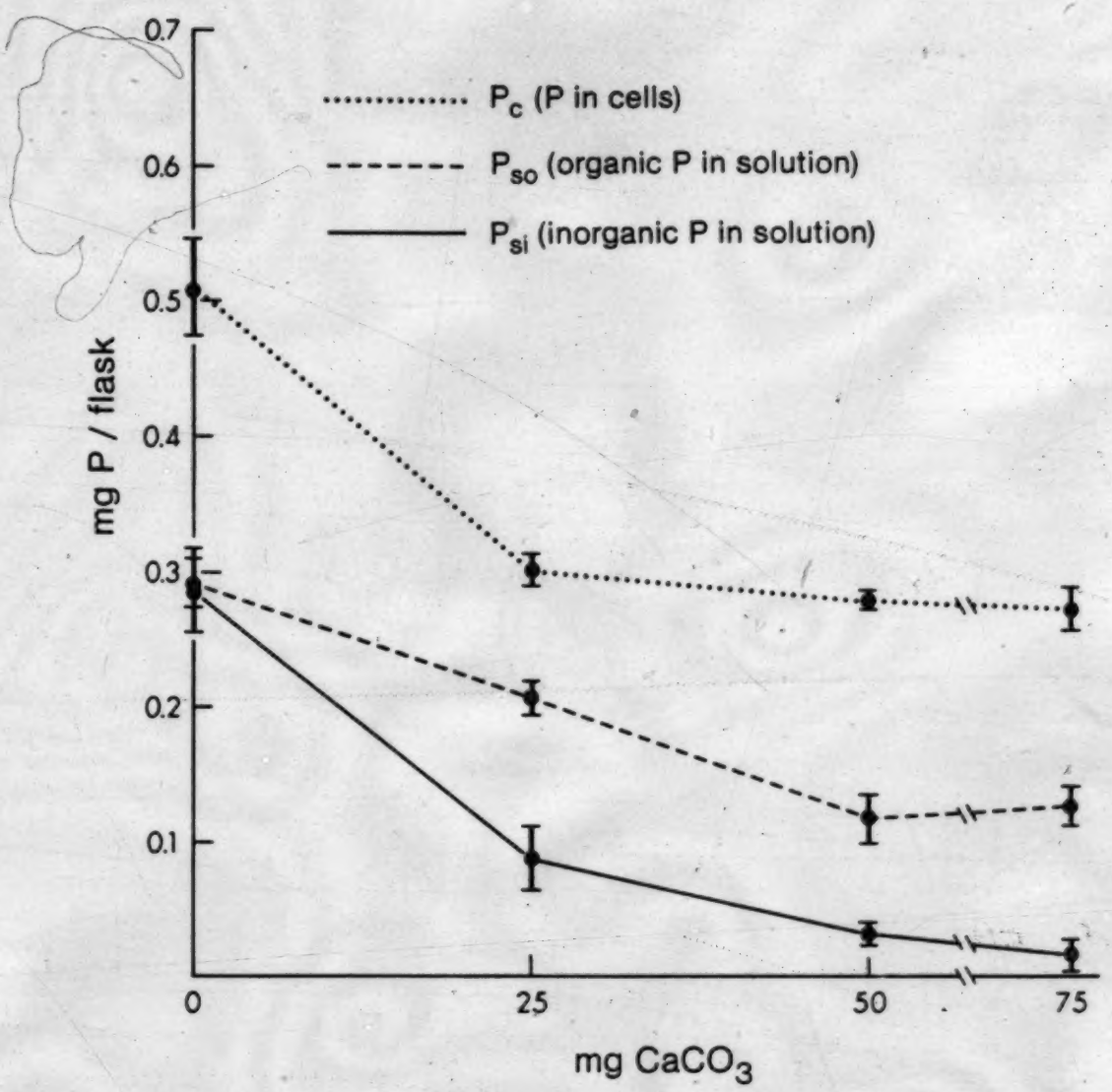

Figure 27. Effect of $CaCO_3$ on solubilization of $Ca_{10}(OH)_2(PO_4)_6$ by culture C_{21} after 10 days incubation with 50 mg $Ca_{10}(OH)_2(PO_4)_6$ /50 ml medium.

Table: 17 % of $CaCO_3$ solubilized by culture C_{14} in the presence of 50 mg $Ca_{10}(OH)_2(PO_4)_6$/50 ml medium.

Weight of $CaCO_3$ added	Ca_i	Ca_i-HA	Ca_i-$CaCO_3$	%$CaCO_3$ solubilized
0.0 mg	12.4 ± 0.42	12.6		
25 mg	19.0 ± 0.16	8.82	10.2	101
50 mg	26.5 ± 0.61	3.54	23.0	115
75 mg	32.8 ± 0.28	2.08	30.7	102

Ca_i = Inorganic Ca^{2+} in solution
Ca_i-HA = Ca^{2+} from hydroxylapatite calculated from the total amount of P dissolved (Table 15) and the Ca/P ratio in hydroxylapatite (2.15).
C_{ai}-$CaCO_3$: Inorganic P in solution from $CaCO_3$ (C_{ai}-C_{ai}-HA)

All values are expressed as mg Ca^{++}/flask.

75 mg $CaCO_3$ / 50 ml medium respectively (Table 18). This is consistant with the earlier observation on the respective effect of these two organisms on pH

Dissolution of $Ca_{10}(OH)_2(PO_4)_6$ and $CaCO_3$ is given by the following equations (Lindsay, 1979) :

$$Ca_{10}(OH)_2(PO_4)_6 \text{ ---- } 10Ca^{2+} + 6PO_4^{3-} + 2OH^-$$

$$CaCO_3 \text{ ---- } Ca^{2+} + CO_3^{2+}$$

In a system containing both $CaCO_3$ and $Ca_{10}(OH)_2(PO_4)_6$, solubility of HA depends upon the activity of Ca^{2+} and on pH. Total concentration of P can be further affected by the formation of metal complexes with one or more phosphate species as a ligand (Bolt and Bruggenwert, 1976).

Introducing Ca^{2+} ions into the system as a result of $CaCO_3$ solubilization by culture C_{14}, shifted the equilibrium of $Ca_{10}(OH)_2(PO_4)_6$ dissolution to the left, thus decreasing solubility of HA and consequently the concentration of P in solution. As the concentration of Ca^{2+} increased from $7*10^{-3}$ to $19.2*10^{-3}$ M, the concentration of phosphorus decreased from $3.35 * 10^{-3}$ to $0.044*10^{-3}$ M (Table 19). This is due to the common ion effect of Ca^{2+}.

Theoretically, concentration of P in solution can be calculated using the following relations (Fritz and Shenk, 1977):

$$Ksp = [Ca^{++}]^{10}[OH^-]^2[PO_4^{---}]^6$$

Table: 18 % of $CaCo_3$ solubilized by culture C_{21} in the presence of 50 mg $Ca_{10}(OH)_2(PO_4)_6$/50 ml medium.

Weight of $CaCO_3$ added	Ca_i	Ca_i-HA	Ca_i-$CaCO_3$	%CaCO3 solubilized
0.0 mg	1.58 ± 0.20	1.21		
25 mg	9.18 ± 0.29	0.450	8.73	91.5
50 mg	8.19 ± 0.82	0.190	8.00	39.9
75 mg	8.23 ± 0.25	0.177	8.05	26.6

Ca_i, Ca_i-HA and Ca_i - $CaCO_3$ as defined in Table 17.

All values are expressed as mg Ca^{++}/flask.

Table 19: Calculated and measured values of M [$H_2PO_4^-$] in solution as affected by M [Ca^{++}]

Culture C_{21}					Culture C_{14}				
mg $CaCO_3$ added	M[Ca^{2+}]	Calc. M[H_2PO^-]	Meas. M[H_2PO^-]	Calc. / Meas.	Mg Ca CO_3 added	M[Ca^{2+}]	Calc. M[H_2PO^-]	Meas. M[H_2PO^-]	Calc. / Meas.
0.0	8.7×10^{-4}	2.9×10^{-8}	2.0×10^{-4}	1.5×10^{-4}	0.0	7.0×10^{-3}	41×10^{-3}	3.4×10^{-3}	12
25	50×10^{-4}	5.1×10^{-10}	0.6×10^{-4}	8.5×10^{-6}	25	10×10^{-3}	2.2×10^{-3}	1.5×10^{-3}	1.5
50	45×10^{-4}	2.4×10^{-9}	0.2×10^{-4}	1.2×10^{-4}	50	14×10^{-3}	1.3×10^{-3}	0.39×10^{-3}	3.3
75	46×10^{-4}	8.5×10^{-10}	0.1×10^{-4}	8.5×10^{-5}	75	19×10^{-3}	0.32×10^{-3}	0.04×10^{-3}	7.3

Calc. = Calculated
Meas. = Measured

$$[PO_4^{***}] = CTP\left(\frac{Ka1.Ka2.Ka3}{[H^+]^3+[H^+]^2Ka1+[H^+]Ka1.Ka2+Ka1.Ka2.Ka3}\right)$$

where:

Ksp = Solubility product $Ca_{10}(OH)_2(PO_4)_6$

CTP = Molar concentration of P in solution

Ka1,Ka2,Ka3 = Dissociation constants of phosphoric acid

To apply the above equations to calculate [P] in the system the following assumptions were made:

1. Activity coefficients of Ca^{2+} and P are unity.
2. Only orthophosphate species contribute significantly to total P in solution.
3. No complex formation occurred.
4. pH and Ca^{2+} concentration are the only controls on dissolution of $Ca_{10}(OH)_2(PO_4)_6$.
5. No unknown side reactions occurred.
6. An equilibrium state was reached.

If one or more of the above assumptions were not met, this would result in an increase or decrease in the calculated value of [P].

Table 19 shows that the calculated values of P in solution of culture C_{14} were 1.5 to 12 times greater than the measured values. In culture C_{21}, however, the calculated values were 4 to 6 orders of magnitude less than the measured values. In culture C_{14} it is likely that the abovementioned assumptions come close to approximating the system and pH and Ca^{2+} concentration are the main controls on concentration of P in solution. In culture C_{21} the situation is fundamentally different and one or more of the

above assumptions are invalid, suggesting that pH and Ca^{2+} concentration alone are not fundamental controls on P concentration in solution for systems such as this where the pH is increased.

To account for the difference between the measured and calculated values of P with culture $C_{2,1}$, the activity coefficient of Ca^{2+} would have to be 10^{-3} or less. For this to be the case most calcium in solution would have to be in a complex form. In culture $C_{2,1}$, however, equilibrium might not be reached because of continuous removal of P from this system by bacterial cells, because the cells did not die by the end of the incubation period.

These results suggest that in calcareous soils and in soil with high buffering capacity, the conversion of sparingly soluble phosphate minerals to microbial P by PDB may be more important than increasing orthophosphate concentration in soil. Microbial P, however, is temporarily held unavailable to plants. Upon decomposition, after the death of the cells, phosphorus may be released in an available form if the organic P is hydrolysed near the root surface.

5.7 Effect of Medium Contents on pH Curves

From the previous data , the pH of culture $C_{1,4}$ was maintained around 4.2 during the experiment (Figure 11). The pH of cultures $C_{2,1}$ and S_4 decreased to 3.9 after one day of

incubation and to 4.5 after four days of incubation for the two organisms respectively, and then increased (Figure 11). Culture C_{21} maintained a pH of 7.3 - 7.9 during the following twenty days of incubation. Culture S_4 maintained a pH of 6.2 - 7.8 during the last seventeen days of incubation.

Whether the pH of the culture is acidic or alkaline appears to depend on whether acids are produced from sugars or alkalis from nitrogen - containing compounds. An organism may lower or raise the pH in response to changing substrates. This hypothesis was examined in a separate experiment. When asparagine was omitted from the medium, the pH of culture C_{21} did not increase after it had been depressed (Figure 28). This is evidence of the influence of substrate on final culture pH, suggesting that both environment and organisms control the final pH.

The effect of the amount of glucose in the medium on pH time course curves was examined using culture C_{21}. In the presence of asparagine, pH of the culture was dependent on the amount of glucose added to the medium (Figure 29). Low concentrations of glucose in the medium (1% or less) were insufficient to produce a low pH (pH 4.0) after two days of incubation and pH increased to more than pH 7.5. When supplied with sufficient amounts of glucose (2% or more), the pH of culture C_{21} was maintained around pH 4.0 after four days of incubation (Figure 16).The characteristic depression followed by an increase was observed at 1%

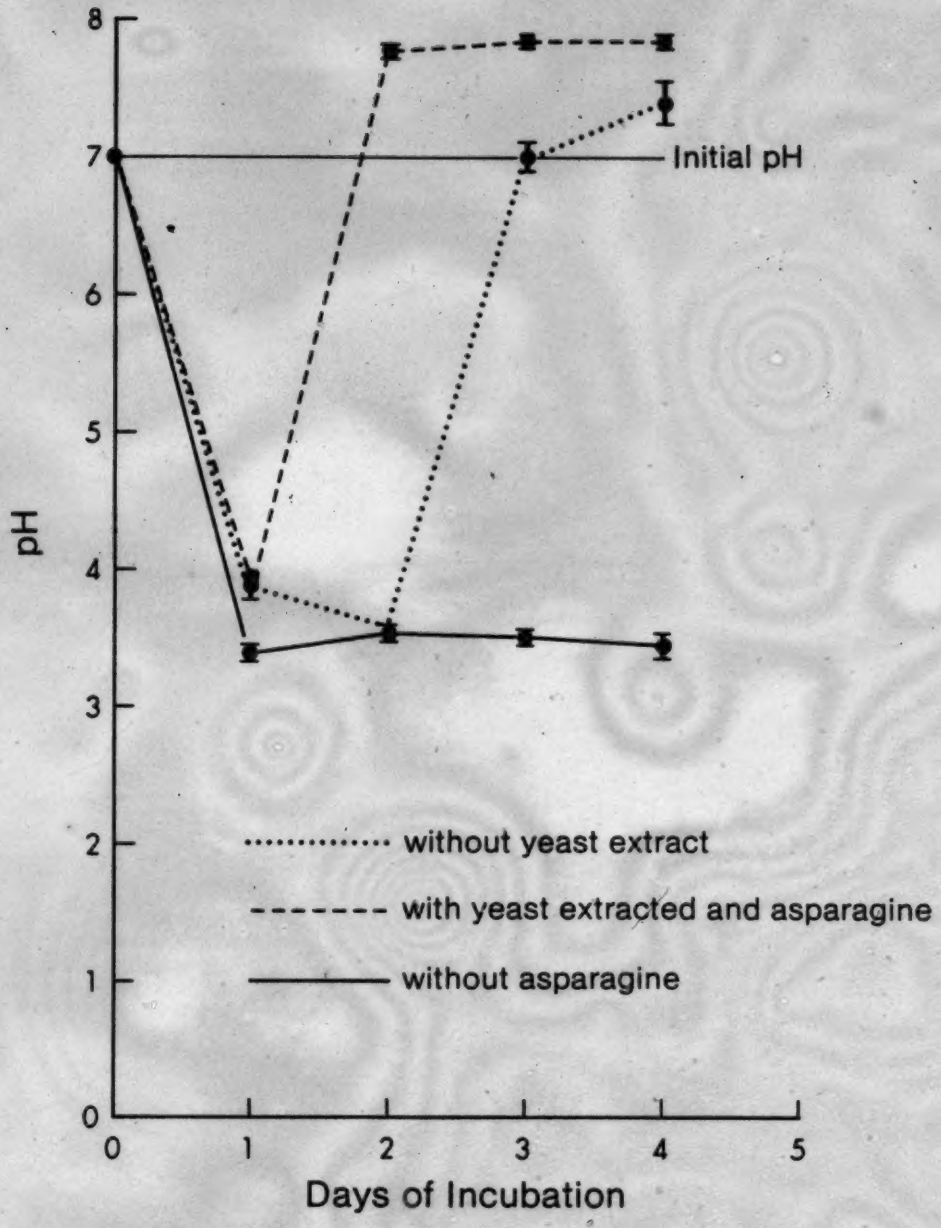

Figure 28. Effect of yeast extract and asparagine on pH curves of culture C_{21} after 4 days of incubation with 50 mg $Ca_{10}(OH)_2(PO_4)_6$/50 ml medium.

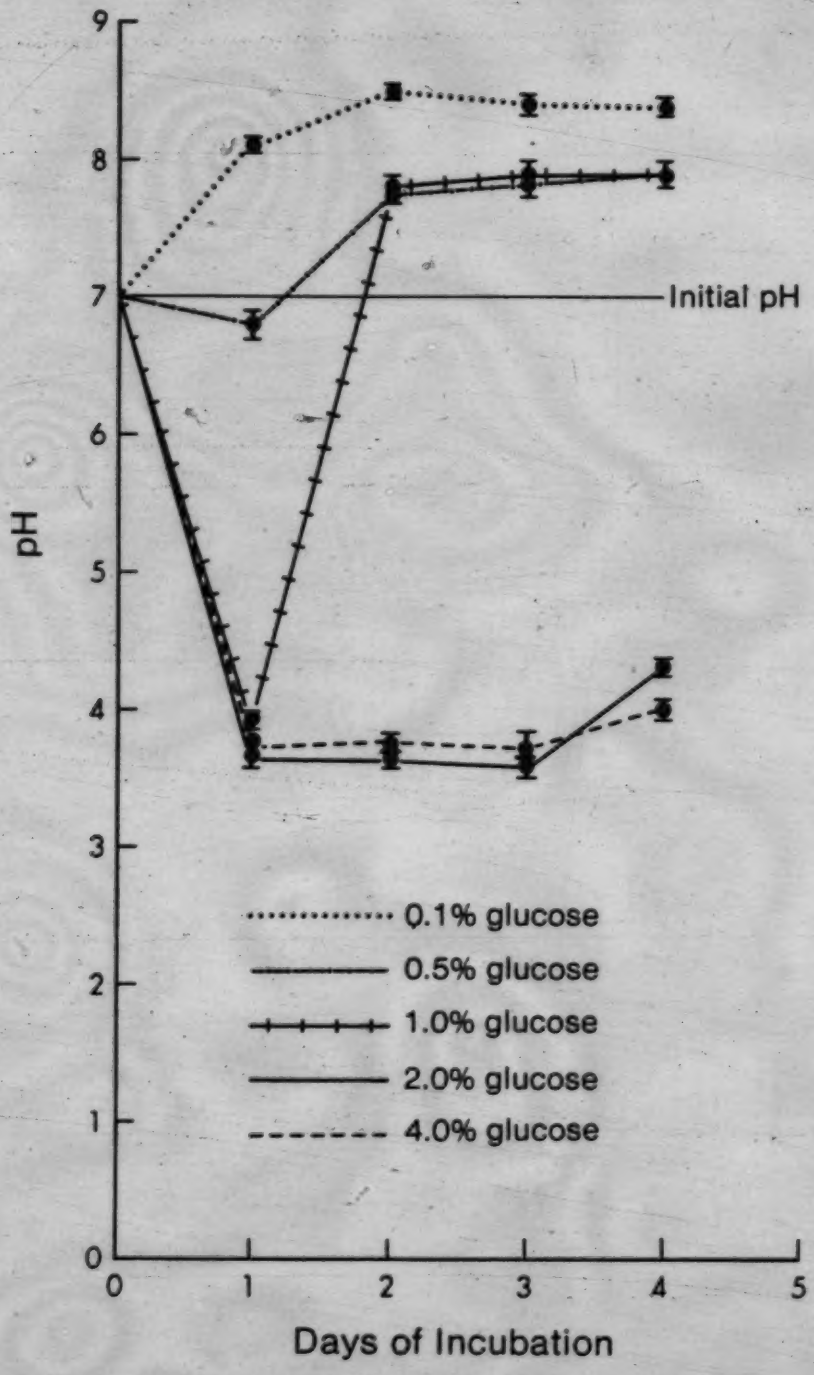

Figure 29. Effect of glucose concentration on pH curves of culture C_{21} after 4 days of incubation with 50 mg $Ca_{10}(OH)_2(PO_4)_6$/50 ml medium.

glucose, which is the concentration used routinely throughout this study. These data suggest a strong influence of the balance of energy - yielding substrates on change in pH and the pH time course curves during growth of an organism capable of utilizing a range of substrates. This may have significant implications in mixed systems such as soil.

Data presented in Appendix 3 further showed a relationship between initial P supply and culture pH. Under limiting - P supply conditions (5 ppm - P), C_{14} failed to lower the pH to less than 6.7 after ten days of incubation. Under higher P supply conditions (25 ppm P or more), however, culture C_{14} lowered the pH to 4.1 after ten days of incubation. A possible explanation is that the initial concentration of 5 ppm P was insufficient to enable the bacteria to oxidize enough glucose to produce the quantity of organic acids necessary to lower the pH. This process, however, seems unrelated to total biomass because the dry weights of C_{14} cells were not significantly different when bacteria were grown in a medium initially containing 5, 25, 65 or 105 ppm of P.

6. Summary and Conclusion

The objectives of this study were to develop a rapid and reliable method for isolation of PDB from soil and to study the transformation of hydroxylapatite $(HA)(Ca_{10}(OH)_2(PO_4)_6$ by PDB in buffered and unbuffered systems.

Gerretsen's (1948) agar plate method (APM) with precipitated phosphate in the medium was modified and used to isolate PDB from soil and to test several pure cultures for their ability to dissolve phosphate minerals. Clear zones around bacterial colonies were taken as indicating their ability to dissolve precipitated phoshate in the medium. When the precipitated phosphate was replaced by HA or $CaHPO_4$, PDB failed to show clear zones around their colonies. This suggested that the clear zone was a secondary reaction, presumably caused by production of organic acids, and not a specific phosphate dissolution mechanism. The organisms which failed to show clearing using the agar plate method (APM) with HA in the medium, dissolved a significant amount of HA in liquid media. These data suggested that Gerretsen's APM with precipated phosphate is more reliable for detecting PDB, and an analytical method measuring the dissolved P is the most appropriate to assess the capacity of PDB to dissolve HA.

A quantitative study to follow the fate of P during dissolution of HA in a liquid medium showed that P dissolved was underestimated when only orthophosphate in solution was

considered, and soluble orthophosphate is not a valid sole criterion to describe the role of bacteria in cycling P from sparingly soluble HA. From the literature one gets the impression that the only important fraction of the dissolved P is P in solution. No reports are available of attempts to draw a phosphorus balance sheet during the dissolution of phosphate minerals by soil microorganisms. In soil, however, the amount of P in solution at any time seldom exceeds 1 ppm. Furthermore, soluble inorganic P in solution might be partially or totally immobilized by microorganisms. Cole *et al*, (1978) found that microbial P increased rapidly during incubation of soil with cellulose and nitrogen. This increase corresponded to a decrease in inorganic P in solution. In a native grassland, the calculated transfer of P ($g\ m^{-2}\ yr^{-1}$) between soil solution and microorganisms was three times larger than the transfer between soil solution and roots (Stewart *et al*, 1980).

To draw a phosphorus balance sheet during dissolution of HA by PDB, orthophosphate in solution (Psi), soluble organic P in solution (Pso) and P in the cells (Pc) must be measured. Measurement of P in the cells required separation of cells from undissolved HA particles. To achieve this, three methods were tested : $NaHCO_3$, Chlorform and HCl. The HCl method was found to be the most reliable and simple one. The effect of HCl on the release of P from cells during dissolution of the remaining undissolved HA particles was investigated using three cultures (S_4, C_{14} and C_{21}). The

amount of P realeased by HCl was found to range from 3 - 20 %, depending on the organism, and was, in most cases, independent of P supply in the medium, P content of the cells and age of the culture if it was sampled after four days of incubation.

Partitioning of P removed from HA during growth of PDB in an unbuffered liquid medium and the effect of PDB on final pH of the medium were examined using 12 PDB cultures. Based on the final pH of the medium, PDB can be grouped into two categories; bacteria which increased the pH of the medium and bacteria which lowered the pH. The group that increased the pH converted more than 80 % of the total P dissolved from $Ca_{10}(OH)_2(PO_4)_6$ into microbial P (Pc and Pso); whereas the group which lowered the pH converted less than 30 % of the total P solubilized into microbial P. In general, the amount of P converted into Pso was greater than that of Pc.

Changes in pH of the medium were characteristic of the medium composition as well as the organism. In the presence of asparagine, the final pH depends on the amount of glucose present. Culture C_{21} failed to raise pH in the presence of 2% or more of glucose in the medium. In the presence of less than 1% glucose, however, culture C_{21} increased pH to more than 7.5. When asparagine was omitted from the medium , the pH of culture C_{21} did not increase even in the presence of less than 1% glucose.

Although lowering pH is beneficial because it solubilizes HA, it has the disadvantage of lowering the activity of bacteria or causing the death of the cells. Culture $C_{1,4}$ died after four days of incubation because of the acidity of the medium.

Addition of $CaCO_3$, as buffering agent, to the medium caused a drastic decrease in the amount of orthophosphate brought into solution from HA (from 4.69 mg to 0.062 mg and from 0.284 mg to 0.014 mg for organisms $C_{1,4}$ and $C_{2,}$ respectively). Microbial P, however, was not significantly affected. These data showed that PDB were able to utilize P from HA and convert it to microbial P regardless of whether the system is buffered or not. Data from this study showed that PDB were able to dissolve HA in the presence of 105 ppm P in the medium, suggesting that dissolution of HA was not dependent on the presence of soluble P.

Microorganisms carry out a large number of phosphorus transformations in soil and at the soil-root interface. The most vital factor affecting plant uptake of P is the rate at which an adequate concentration of P is returned to the soil solution at the root surface. Daily root uptake from solution during periods of high root activity and near ideal temperature and water status may be fifty times the amount of P in solution (Cole *et al* ,1977). Replenishment of soluble P in the depleted zone depends on;

1. the release of P from slightly soluble phosphate minerals

2. desorption from phosphate adsorbing surfaces
3. mineralization of organic P.

Microbial transformations of labile and stable organic P often produce the majority of the plant available solution P (Paul and Vorney,in press).

The possible role of PDB in maintaining a high concentration of P in solution at the root surface lies in their ability to increase solubility of sparingly phosphate minerals. Taking HA as an example, its solubility can be represented as follows :

$Ca_{10}(OH)_2(PO_4)_6$-----$10Ca^{2+} + 6PO_4^{3-} + 2OH^-$

Removal of any of the products will result in increasing HA solubility. Presence of a large population of PDB in the rhizosphere and rhizoplane of plants along with high amounts of energy compounds from root exudates would alter pH around and at the root surface. The pH of the rhizosphere and rhizoplane is dynamic and depends on the nature of root exudates which PDB utilize as substrate. The use of carbohydrate results in production of organic acids, thus lowering the pH and increasing solubility of phosphate minerals. Both plants and microorganisms compete for P in solution. Bacteria, however, accumulate more P (1.5 - 2.5% of their dry weight) than plants (0.05 - 0.5% of their dry weight). Stewart *et al*,(1980) found that the immobilization of inorganic P as microbial P approximated 64 ug g^{-1} soil (140 kg P ha^{-1} in the top 15 cm) in five days following the addition of cellulose. The immobilized microbial P may be

released in available form after the death of the cells. The argument that soil is too highly buffered with respect to pH to permit such increase in P concentration might not hold in the root region because of high microbial activity. If, however, PDB pump P into a labile P pool, which was shown to be the case even in a buffered system, PDB may play an unsuspected role in P cycling and plant nutrition.

PDB use protein as a substrate and the subsequent production of ammonia could result in a higher pH which might cause phosphate precipitation.

From this study we can conclude the following :

1. APM with precipitated phosphate in the medium is a reliable and simple method for the routine isolation of PDB from soil.
2. Orthophosphate in solution is not a valid sole criterion to assess the role of PDB in cycling P minerals and microbial P should be considered.
3. pH is the main control of P concentration in solution
4. The total amount of P transformed (P in solution and microbial P) is a characteristic of each organism, and pH is not the main factor that controls transformation.
5. Zero P or a depletion of P in solution does not mean that the organisms are unable to transform HA. A certain PDB might dissolve large amounts of P and consume them all.
6. pH at the end of the incubation period gives no information about pH during that period. An organism may

lower pH during the first few days of incubation and solubilize P during that period. The solubilized P may be totally or partially consumed as the pH increased and as the activity of the bacteria increasd. The dissolved P might also re-precipitate as the pH becomes alkaline. The effect of an organism on final pH is a function of both medium composition and the organism itself.

7. The ability of PDB to transform HA was not influenced by the supply of soluble P.
8. PDB play an important role as both source and sink of phosphorus.
9. Phosphorus, once incorporated into biomass, may be released through cell death in the absence of predators or other lytic organisms.

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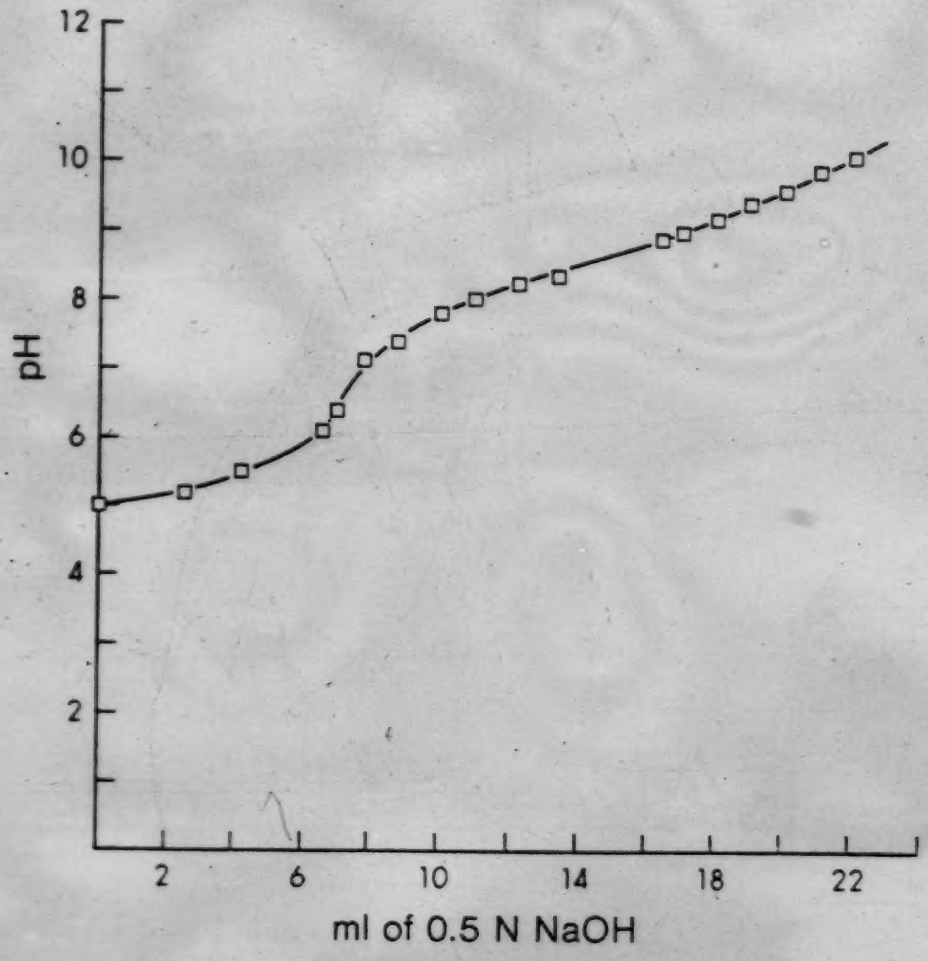
APPENDICES

Appendix 1



Titration curve for agar medium with precipitated phosphate (prepared by mixing 15 ml of 10% sterilized K_2HPO_4 and 25 ml of 10% sterilized $CaCl_2$ with 500 ml of liquid medium).

Appendix 2

Loss by evaporation from 250 ml erlenmeyer flasks during 21 days of incubation on a rotary shaker at 250 rpm. Original volume was 50 ml.

Days of incubation	ml lost
1	0.43
2	1.20
3	1.65
4	2.13
5	2.41
6	2.97
7	3.56
10	4.81
12	5.86
15	7.11
21	9.25

Appendix 3

P uptake, dry weight, % P in bacteria and the effect of HCl on the release of P from the cells.

a. P uptake, dry weight, % P in bacteria and the effect of HCl on the release of P from the cells of different bacterial cultures after 10 days of incubation with different P concentrations in the medium. Volume of the original medium was 50 ml.

Culture C_{14}:

Initial P (ppm)	pH	Psi	Pso	Pc-PH	PH	Pc	%PH	PO (Pc+Pso)	BW	%P
05	6.7 ± 0.13	Nil	0.249 ± 0.028	0.145 ± 0.001	0.054 ± 0.002	0.199	27	0.448	24.1 ± 0.603	0.830
25	4.0 ± 0.05	0.130 ± 0.007	0.725 ± 0.020	0.561 ± 0.025	0.024 ± 0.001	0.585	4	1.31	26.6 ± 1.36	2.19
65	4.1 ± 0.00	2.20 ± 0.029	0.656 ± 0.042	0.552 ± 0.003	0.025 ± 0.002	0.577	4	1.23	22.9 ± 1.27	2.52
105	4.1 ± 0.03	4.01 ± 0.310	0.624 ± 0.011	0.629 ± 0.008	0.026 ± 0.001	0.655	4	1.29	26.8 ± 2.08	2.45

Culture C_{21}

Initial P (ppm)	pH	Psi	Pso	Pc-PH	PH	Pc	%PH	PO (Pc+Pso)	BW	%P
05	8.3 ± 0.08	0.001 ± 0.0001	0.125 ± 0.020	0.148 ± 0.007	0.071 ± 0.001	0.219	32.4	0.364	30.7 ± 3.33	0.713
25	8.0 ± 0.03	0.719 ± 0.018	0.136 ± 0.022	0.441 ± 0.014	0.083 ± 0.004	0.524	15.8	0.659	53.6 ± 1.26	0.978
65	7.8 ± 0.03	2.11 ± 0.046	0.215	0.481 ± 0.049	0.113 ± 0.006	0.594	19.0	0.800	58.3 ± 2.62	1.02
105	7.4 ± 0.03	3.75 ± 0.137	0.260	0.517 ± 0.027	0.118 ± 0.012	0.635	18.6	0.895	56.1 ± 0.854	1.13

Culture S_4:

Initial P (ppm)	pH	Psi	Pso	Pc-PH	PH	Pc	%PH	PO (Pc+Pso)
25	8.0 ± 0.06	0.020 ± 0.002	0.294 ± 0.011	0.720 ± 0.073	0.090 ± 0.002	0.810	11.1	1.11
65	7.9 ± 0.28	0.400 ± 0.013	1.16 ± 0.030	1.11 ± 0.040	0.102 ± 0.004	1.21	8.39	2.37
105	7.5 ± 0.00	2.59 ± 0.500	1.53 ± 0.000	1.07 ± 0.058	0.138 ± 0.005	1.21	11.4	2.74
505	7.3 ± 0.05	19.70 ± 3.20	1.90 ± 0.000	0.841 ± 0.036	0.138 ± 0.015	0.979	14.1	2.88

Psi : Inorganic soluble P
Pso : organic soluble P
PH : P released by HCl
Pc : Biomass - P
Po : P uptake (assuming that no P was released after it had been taken).
$\%PH = \frac{PH}{Pc} \times 100$
Bw = Biomass dry weight
%P = Percentage of P in bacteria (dry basis)

All values are expressed as mg P/flask.

Appendix 3

b. P uptake, dry weight, % P in the cells and the effect of HCl on the release of P from the cells of different bacterial culture after different times of incubation. Concentration of P in the original medium was 105 ppm and the initial volume was 50 ml.

Culture C_{14}

Days	pH	Psi	Pso	Pc-PH	PH	Pc	%PH	$\frac{PH}{(Pc+Pso)}$	DW	%P
1	4.6 ± 0.03	4.26 ± 0.077	0.155 ± 0.003	0.388 ± 0.036	0.094 ± 0.011	0.482	19.5	0.637	21.5 ± 1.29	2.24
2	4.3 ± 0.03	4.17 ± 0.075	0.405 ± 0.019	0.517 ± 0.006	0.109 ± 0.009	0.626	17.4	1.13	29.3 ± 1.39	2.14
3	4.1 ± 0.04	4.19 ± 0.148	0.443 ± 0.003	0.460 ± 0.029	0.087 ± 0.002	0.547	15.9	0.990	26.3 ± 1.39	2.08
4	4.1 ± 0.07	4.16 ± 0.116	0.507 ± 0.054	0.350 ± 0.039	0.032 ± 0.002	0.382	8.38	0.889	24.3 ± 1.88	1.57
7	4.3 ± 0.04	4.31 ± 0.069	0.380 ± 0.021	0.442 ± 0.006	0.047 ± 0.003	0.489	9.61	0.869	-	-
10	4.2 ± 0.03	4.21 ± 0.097	-	0.436 ± 0.009	0.015 ± 0.001	0.451	3.33	-	-	-
12	4.1 ± 0.03	4.21 ± 0.080	0.477 ± 0.024	0.419 ± 0.007	0.016 ± 0.002	0.435	3.68	0.912	22.5 ± 0.465	1.93
15	4.3 ± 0.02	4.22 ± 0.292	0.264 ± 0.036	0.409 ± 0.041	0.014 ± 0.001	0.413	3.34	0.677	23.4 ± 1.24	1.73
21	4.2 ± 0.04	4.36 ± 0.065	-	0.402 ± 0.023	0.016 ± 0.003	0.418	3.83	-	22.6 ± 0.915	1.85

Culture C_{21}

Days	pH	Psi	Pso	Pc-PH	PH	Pc	ΣPH	PO (Pc+Pso)	BW	ΣP
1	3.9 ± 0.03	3.62 ± 0.032	0.350	0.660 ± 0.080	0.139 ± 0.002	0.799	17.4	1.15	43.4 ± 0.557	1.84
2	7.8 ± 0.03	3.31 ± 0.021	0.418 ± 0.025	0.820 ± 0.041	0.172 ± 0.020	0.992	17.3	1.33	62.4 ± 3.49	1.59
3	7.9 ± 0.05	3.58 ± 0.056	0.375	0.704 ± 0.110	0.147 ± 0.004	0.851	17.3	1.23	54.8 ± 2.31	1.55
4	7.9 ± 0.05	3.80 ± 0.031	0.325	0.646 ± 0.018	0.133 ± 0.009	0.779	17.1	1.20	51.4 ± 1.01	1.52
7	7.7 ± 0.03	3.91 ± 0.017	0.340 ± 0.038	0.521 ± 0.027	0.096 ± 0.004	0.617	15.6	0.957	50.0 ± 1.38	1.23
10	7.3 ± 0.03	4.12 ± 0.059	0.330 ± 0.014	0.390 ± 0.021	0.081 ± 0.001	0.471	17.2	0.796	48.9 ± 1.20	0.96
12	9.3 ± 0.00	4.33 ± 0.031	0.250 ± 0.035	0.345 ± 0.011	0.064 ± 0.005	0.409	15.7	0.759	43.2 ± 1.87	0.95
15	7.3 ± 0.00	4.30 ± 0.120	0.215	0.279 ± 0.001	0.053 ± 0.001	0.333	15.9	0.548	-	-
21	7.3 ± 0.00	4.33 ± 0.031	0.130	0.231 ± 0.014	0.019 ± 0.003	0.250	7.6	0.380	38.8 ± 1.47	0.64

Culture S_4

Days	pH	Psi	Pso	Pc-PH	PH	Pc	ΣPH	PO (Pc+Pso)
1	7.5 ± 0.10	4.89 ± 0.438	0.115 ± 0.007	0.132 ± 0.041	0.017 ± 0.006	0.149	11.2	0.264
2	6.9 ± 0.12	3.64 ± 0.183	0.140 ± 0.014	1.03 ± 0.072	0.318 ± 0.020	1.35	23.6	1.49
4	4.5 ± 0.06	2.40 ± 0.315	0.360 ± 0.085	1.94	0.339	2.27	14.9	2.63
7	6.2 ± 0.15	2.35 ± 0.034	0.340 ± 0.110	1.74 ± 0.092	0.321 ± 0.005	2.06	15.9	2.40
10	7.0 ± 0.06	2.40 ± 0.144	0.250 ± 0.007	1.62 ± 0.052	0.312 ± 0.003	1.93	16.2	2.18
15	7.7 ± 0.29	2.38 ± 0.000	0.840 ± 0.042	0.983 ± 0.095	0.226 ± 0.029	1.21	18.7	2.05
21	7.8 ± 0.14	2.15 ± 0.028	0.971 ± 0.100	0.725 ± 0.039	0.246 ± 0.044	0.971	25.3	1.94

Psi, Pso, P_H, Pc, Po, ΣP_H, RW and ΣP as defined in (a)

All values are expressed as mg P/flask.

Appendix 5

Solubilization of $Ca_{10}(OH)_2$ $(PO_4)_6$ by several PDB and fate of P dissolved following 10 days of incubation with 50 mg $Ca_{10}(OH)_2$ $(PO_4)6$/50 ml medium. All values or expressed as mg P/flask unless otherwise stated.

Organism	pH	Psi	Psi (Mole/L) X10-3	Pso	Pc	Total P dissolved	%P dissolved	%P recovered	Po (Pso + Pci)	% Po of the Total dissolved
S_1	4.4 ± 0.05	4.23 ± 0.18	3.03	1.02 ± 0.16	0.116 ± 0.016	5.42	57.6	94.1	1.19	22
S_2	4.3 ± 0.05	5.99 ± 0.65	4.29	0.315 ± 0.02	0.296 ± 0.032	6.60	57.2	91.5	0.611	9.3
S_3	4.1 ± 0.00	4.74 ± 0.16	3.39	1.20 ± 0.13	0.726 ± 0.03	6.67	70.8	110	1.93	29
S_4	8.4 ± 0.05	0.254 ± 0.013	0.181	0.776 ± 0.10	1.00 ± 0.05	2.03	21.9	96.0	1.78	88
S_5	4.9 ± 0.05	3.19 ± 0.035	2.28	-	0.521 ± 0.003	3.70	40.9	103	0.521	14
S_6	4.4 ± 0.05	5.22 ± 0.08	3.74	0.352 ± 0.026	0.161 ± 0.007	5.74	54.5	98.5	0.513	11
C_3	4.7 ± 0.06	1.97 ± 0.07	1.41	-	0.574 ± 0.047	-	-	-	-	-
C_5	4.7 ± 0.00	3.22 ± 0.19	2.30	-	0.606 ± 0.011	3.83	41.0	100	0.606	16
C_{14}	4.3 ± 0.00	5.62 ± 0.10	4.03	0.216 ± 0.03	0.262 ± 0.008	6.10	64.9	107	0.478	10
C_{21}	7.8 ± 0.38	0.218 ± 0.008	0.155	0.43 ± 0.02	0.440 ± 0.003	1.09	11.8	88.0	0.870	80
C_{22}	4.2 ± 0.1	3.22 ± 0.18	2.30	0.13 ± 0.01	0.068 ± 0.0008	3.42	36.3	80.0	0.198	5.8
C_{25}	3.1 ± 0.05	8.00 ± 0.12	5.71	-	0.728 ± 0.024	-	-	-	-	-

Psi, Pso, Pc, Po as defined in Table 14

Appendix 6

Time and rpm required to settle hydroxylapatite particles and bacterial cells by centrifugation.

The following equation was used (Whittig 1965):

$$t = \frac{n \log (R/S)}{3.81 \times N^2 \times r^2 \times (\Delta S)}$$

Where:

t : time in second

R: the radius in cm. of rotation of the top of the sediment in the tube.

S: the radius in cm. of rotation of the surface of the suspension in the tube.

N: revolutions per second

r: particle radius in cm.

n: vescosity of the liquid in poises at the existing temperature.

(ΔS): difference between specific gravity of the particle and that of the suspension liquid.

Time was set up to be 20 minutes:

R = 10 cm.

S = 6 cm.

$n = 1.8 \times 10^{-2}$ poises at 0°C

density of bacterial cells = 1.1 g/cm^3

density of hydroxylapatite particles = 2.6 g/cm^3

radius of bacterial cells = 0.2 μm

radius of hydroxylapatite particles = 1 μm

Calculating rpm required to settle bacterial cells after 20 minutes.

$$1200 = \frac{1.8 \times 10^{-2} \times \log (\frac{10}{6})}{3.81 \times N^2 \times (2 \times 10^{-5})^2 \times 0.1}$$

$$N^2 = \frac{0.3996 \times 10^{-2}}{18288 \times 10^{-11}}$$

$N^2 = 2.19 \times 10^{-4}$

$N = 148$ rps

$N = 8880$ rpm

Because the size of the cells varies, the contents of the tube were centrifuged at 12000 rpm or R.C.F. = 17300

Using the same equation, rpm required to settle hydroxylapatite particles = 510 rpm. The actual speed used was 1000 rpm on R.C.F. = 121.